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Mechanisms to prevent the toxicity of chronic neuroinflammation on forebrain cholinergic neurons

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Abstract

Inflammatory processes may play an important role in the degeneration of basal forebrain cholinergic cells Alzheimer's disease. We infused the proinflammagen lipopolysaccharide into the basal forebrain of young rats and determined whether the chronic administration of two novel non-steroidal anti-inflammatory drugs or a pan-caspase synthesis inhibitor, z-Val–Ala–Asp(OMe)-fluoromethyl ketone (zVAD), could provide neuroprotection from the cytotoxic effects of the neuroinflammation. Chronic lipopolysaccharide infusions decreased choline acetyltransferase activity and increased the number of activated microglia within the basal forebrain region. The level of caspases 3, 8 and 9 was increased in ventral caudate/putamen. Non-steroidal anti-inflammatory drug therapy attenuated the toxicity of the inflammation upon cholinergic cells and reduced caspases 3, 8 and 9 activity in the caudate/putamen. zVAD treatment significantly decreased the levels of caspases 3, 8 and 9 but did not provide neuroprotection for the cholinergic neurons. These results suggest that prostaglandins contribute to the degeneration of forebrain cholinergic neurons in Alzheimer's disease.

Keywords: Neuroinflammation; (Rat); Forebrain basal; Acetylcholine; Caspase; Non-steroidal anti-inflammatory drug

1. Introduction

Alzheimer's disease is characterized by specific neuropathological changes and a forebrain deficiency of acetylcholine (Davis et al., 1999; Whitehouse et al., 1981). The decline in the number of cholinergic cells within the basal forebrain may contribute to aspects of the cognitive impairments associated with Alzheimer's disease (McGeer et al., 1984; Muir, 1997). Although chronic inflammatory processes may play an important role in the pathogenesis of Alzheimer's disease (Akiyama et al., 2000; Aisen and Davis, 1994; Griffin et al., 1998; McGeer et al., 1989; McGeer and McGeer, 1999; Mrak et al., 1995; Rogers et al., 1992; Rogers, 1995; Tan et al., 1999), the mechanism underlying the degeneration of basal forebrain cholinergic cells is unknown. Alzheimer's disease is associated with increased levels of inflammatory cytokines, such as interleukin-1 and tumor necrosis factor-alpha (Bauer et al., 1991; Griffin et al., 1989; McGeer and McGeer, 1998) as well as various caspases (Shimohama et al., 1999) that could contribute to the neurodegenerative process (Akiyama et al., 2000; Blasko et al., 1997). A potential role for neuroinflammation, and the specificity of its effects upon cholinergic neurons, was suggested by a study that isolated antibodies from the sera of Alzheimer's disease patients that selectively recognized and destroyed basal forebrain cholinergic cells when injected into a rat brain (Foley et al., 1988). In addition, head trauma in humans is a significant risk factor for Alzheimer's disease (Rasmusson et al., 1995) and is associated with increased

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levels of inflammatory proteins (Griffin et al., 1994) and a decline in the number of basal forebrain cholinergic neurons (Murdoch et al., 1998).

Chronic, regional brain inflammation and selective cholinergic neuronal degeneration can be produced by infusion of the proinflammagen lipopolysaccharide into the basal forebrain of young rats (Wenk and Willard, 1999; Willard et al., 1999). In vitro studies also indicate that brain inflammation may selectively destroy basal forebrain cholinergic neurons (McMillian et al., 1995). Lipopolysaccharide can stimulate the endogenous production of caspases, cytokines and other inflammatory cytokines by activated glia (Bluthe et al., 1992; Quan et al., 1994). The current study investigated whether cholinergic neurons within the basal forebrain could be rescued from the cytotoxic effects of chronic lipopolysaccharide infusion by treatment with two novel cyclooxygenase inhibitors that lack gastrointestinal toxicity, i.e. nitric oxide (NO)-flurbiprofen (2-fluoro-a-methyl[1,1′-biphenyl]-4-acetic acid, 4-(nitrooxy)butyl ester) and NCX 2216 (*trans* 3-4[4-[2-fluoro-α-methyl- 1(1,1′-biphenyl)-4-acetyloxy]-3-methoxyphyenyl]-2-propenoic acid 4-(nitrooxy)butyl ester) (Nicox, France). NO-flurbiprofen and NCX 2216 are derivatives of flurbiprofen, an inhibitor of cyclooxygenase enzymatic activity (IC_{50} type $1/IC_{50}$ type $2 = 10.27$), that are effective within the brain following peripheral administration at these doses (Hauss-Wegrzyniak et al., 1998, 1999). NO-flurbiprofen and NCX2216 are novel non-steroidal anti-inflammatory drugs that show a significant attenuation of the gastrointestinal side effects (Fiorucci et al., 1999a; Wallace et al., 1994). These drugs were produced by the incorporation of a NO moiety through an ester linkage to the carboxyl group of an anti-inflammatory drug. NO plays an important role in gastric mucosal defense; drugs that generate NO reduce the severity of gastric mucosal injury in vivo and ex vivo (Kitagawa et al., 1990; MacNaughton et al., 1989). The rationale underlying the development of these compounds was the recognition that the NO released from these derivatives would prevent the pathogenic events that occur subsequent to the suppression of prostaglandin synthesis, in particular the reduced blood flow to the gastric mucosa. Previous experiments have demonstrated that these novel NO-non-steroidal anti-inflammatory drugs have good anti-inflammatory efficacy and gastrointestinal tolerability in rats (Fiorucci et al., 1999a,b; Wallace et al., 1994). We have also determined previously that daily peripheral administration of NO-flurbiprofen can significantly reduce the number of activated microglial cells in young rats (Hauss-Wegrzyniak et al., 1998, 1999).

Finally, the current study also investigated whether the generation of caspase activity contributes to the degeneration of basal forebrain cholinergic cells. Lipopolysaccharide-infused rats were given chronic therapy with a potent and irreversible pan-caspase synthesis inhibitor, z-Val–Ala–Asp(OMe)-fluoromethyl ketone (zVAD, Harada and Sugimoto, 1998).

2. Material and methods

2.1. Subjects

Fifty young (3 months, 250 g) male Fisher-344 rats (Harlan Sprague–Dawley) were housed in pairs in a colony room with a 12:12 dark/light cycle with lights off at 10:00 and food and water provided ad libitum.

2.2. Surgical procedures

Each rat was anesthetized with pentobarbital (50 mg/kg, ip), given 0.3 ml of atropine methylbromide (5 mg/ml, ip), and placed in a stereotaxic instrument with the incisor bar set 3 mm below the earbars (i.e. flat skull). The scalp was incised and retracted and holes were drilled in appropriate locations in the skull with a dental drill. An Alzet (Palo Alto, CA) osmotic minipump (model 2004; 0.25 μl/h) containing lipopolysaccharide (Sigma, St. Louis, MO, *Escherichia coli*, serotype 055:B5, TCA extraction, 1.0 μg/μl) was implanted into the dorsal abdomen and attached via Tygon tubing (0.060 in. O.D.) to a chronic indwelling cannula (Model 3280P, osmotic pump connect, 28 gauge, Plastics One, Roanoke, VA) that have been positioned stereotaxically so that the cannula tip extended into the basal forebrain at the following coordinates: AP: −0.6 mm, ML: +2.8 mm, and DV: −7.9 mm (from the skull). Controls were infused with the artificial cerebrospinal fluid (aCSF) vehicle: (in mM) 140 NaCl; 3.0 KCl; 2.5 $CaCl_2$; 1.0 $MgCl_2$; 1.2 Na_2HPO_4; pH 7.4. The 2004 minipump delivered 0.25 μl/h for at least 37 days (as estimated by information provided by Alzet).

The following five groups of 10 rats were prepared: (1) CSF-infused rats; (2) lipopolysaccharide-infused rats that were injected daily (sc) with drug vehicle (1.0 ml/kg/day); (3) lipopolysaccharide-infused rats that were injected daily (sc) with NO-flurbiprofen (15 mg/kg/day); (4) lipopolysaccharide-infused rats that were injected daily (sc) with NCX 2216 (7.5 mg/kg/day); (5) lipopolysaccharide-infused rats that were also co-infused via the same minipump with zVAD (27 nmol/kg/day). The concentration of zVAD in the osmotic minipumps was 1.2 mM. Therefore, the osmolarity of infusion solution was changed only slightly by the presence of the drug. Also, the drug was dissolved into the same solution as the lipopolysaccharide and therefore it did not alter the total infusion volume. The drug vehicle for the two NO-non-steroidal anti-inflammatory drugs was dimethylsulfoxide/ethanol/castor oil, 20:5:75. We have previously shown that this vehicle does not influence brain inflammation (Hauss-Wegrzyniak et al., 1998).

During the infusion period, each animal's body weight was determined daily and behavior was monitored for indications of drug toxicity. For all rats, post-operative care included chloramphenicol (1% solution) applied to the exposed skull and scalp prior to closure, lidocaine applied

locally to the scalp to lessen pain, and 5.0 ml of sterile isotonic saline injected (sc) to prevent dehydration. All efforts were made to minimize discomfort or pain to the rats. The rats were closely monitored during recovery and kept under a heat lamp or on a heating pad until they were awake and active.

2.3. Biochemical studies

Thirty-seven days after minipump implantation, six rats from each group were deeply anesthetized and sacrificed by decapitation. Tissue samples were taken from right and left basal forebrain of six rats from each group and then assayed for choline acetyltransferase activity. The basal forebrain region was isolated by making three scalpel cuts: a vertical cut near the lateral border of the hypothalamus (inferior from the lateral ventricle), a horizontal cut made through the ventral pallidum just above the level of the anterior commissure and a diagonal cut to remove the most ventral edge of cortex. Choline acetyltransferase activity in the cortex was measured by the formation of [^{14}C]acetylcholine formed from [1-^{14}C]acetylcoenzyme-A and choline (Fonnum, 1969). The choline acetyltransferase enzyme is specific for cholinergic cells; its decline is used as a standard measure of cholinergic cell loss in the basal forebrain (Wenk et al., 1992, 1994). Protein content was determined for each sample by a standard method (Lowry et al., 1951). All assays were performed in triplicate. Choline acetyltransferase enzymatic data were analyzed by analysis of variance (ANOVA) using Student–Newman–Keuls post-hoc analyses.

Lipopolysaccharide, or the consequences of its actions upon glia and neurons, diffused throughout the basal forebrain region, including the ventral striatum, and also into both sides of the brain. The histological studies indicate that activated microglia can be seen on both sides of the brain. Therefore, tissue samples containing the caudate/putamen were taken bilaterally from a region directly dorsal to the basal forebrain sample. These samples were assayed for endogenous levels of caspase 3, 8 and 9 (Fiorucci et al., 1999a,b). Caspases are a large family of molecules that are cysteine proteases; these enzymes cleave proteins after aspartitic acid residues in cells that are undergoing apoptosis (Alnemli et al., 1996). Caspases 8 and 9 are considered initiating caspases and are vital to the pathway of that leads to cell death via activation of an effector caspase, such as caspase 3 (Chaudhary et al., 1999; Hakem et al., 1998; Namura et al., 1998). It has been suggested that increased caspase activity may underlie the selective neurodegenerative changes seen in Alzheimer's disease (Masliah et al., 1998).

For detection of activity for caspases 3, 8, and 9, brain sample homogenates (100–150 mg) were immediately frozen at −80°C in liquid nitrogen. Samples were then placed in a liquid nitrogen while mechanically pulverized and, the resultant powder reconstituted into ice-cold lysis buffer containing (in mmol/l): 50.0 Tris–HCl pH 8.0, 150.0 NaCl, 1.0 EGTA pH 8.0, 100.0 NaF, 10% glycerol, 1.0 $MgCl_2$, 1% v/v Triton × 100, 1.0 Na_3VO_4, 1.0 phenylmethyl sulfonyl fluoride, 10.0 μg/ml leupeptin and 5.0 μg/ml aprotinin at 4°C for 15 min, followed by centrifugation at 20,000 × *g*, for 2 min (Fiorucci et al, 1999a,b). Caspase 3 (CPP-32)-like activity was detected in resulting supernatants by measuring the proteolytic cleavage of the fluorogenic substrate 7-amino-4-trifluoro-methylcoumarin (AFC)-DEVD and AFC as standard in the assay buffer (100 mM HEPES, 10% sucrose, pH 7.5, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTT) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Similarly, caspase 1 (ICE)-like activity was assayed by measuring the proteolytic cleavage of 7-amino-4-coumarin (AMC)-conjugated YVAD-peptide as substrate and AMC as standard at an excitation wavelength of 380 nm vs. an emission wavelength of 460 nm. Caspase 8 (FLICE)-like activity was measured using IEDT-AFC substrate and AFC as standard at an excitation wavelength of 400 nm vs. an emission wavelength of 505 nm. All assays were carried out in a Hitachi 2000 (Pabisch, Milan, Italy) fluorimeter. Brain homogenate supernatants (1 mg of protein) were pre-incubated for 15 min at 37°C to ensure equilibrium, and the reaction, initiated by addition of 14 μM YVAD-AMC, DEVD-AFC or IEDT-AFC alone or in combination with 10 μM of specific inhibitors (Ac.YVAD-CHO, AC.DEVD.CHO or Z-I-E (OMe)-T-D-(OMe)-FMK) and was followed for 20 min. Substrates and inhibitors were added in dimethylsulfoxide, whose final concentration never exceeded 1% and had no effect on enzymatic activity. Protein content was analyzed using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). The specific activity of caspase 3 and 8, 9-like protease in brain homogenates was 0.9 ± 0.4, 1.1 ± 0.5 and 0.7 ± 0.5 pmol AFC/mg protein/min, respectively (four samples each one assayed in duplicate). Caspase data were analyzed by one-way ANOVA.

2.4. Histological studies

Approximately 37 days after minipumps were implanted, the remaining four rats from each group were used for histological analysis and were deeply anesthetized and then prepared for analysis by in situ perfusion of the brain with cold saline containing heparin. The brain was perfused with filtered 4% paraformaldehyde in 0.1 M Na^+ phosphate buffer, pH 7.4, post-fixed in 4% paraformaldehyde/0.1 M Na^+ phosphate buffer overnight, cryoprotected in 0.1 M Na^+ phosphate buffer containing 20% sucrose at 4°C for 24 h, snap-frozen by transfer into isopentane (−50°C), and stored then at −70°C. Coronal sections (40 μm) that included the entire extent of the basal forebrain of both hemispheres were cut on a cryostat for histological analysis. Activated microglia rapidly up-

Table 1
Levels of choline acetyltransferase activity (nmol h^{-1} mg^{-1} protein) in the injected (left) basal forebrain region, as compared to the un-injected (right) side

	Left NBM	Right NBM	% Decline
CSF	88.58 ± 5.24	91.41 ± 7.03	2.8
LPS	67.98 ± 4.29	91.35 ± 4.33	25.5[a]
LPS + NFP	75.89 ± 3.11	83.81 ± 4.93	9.2
LPS + NCX2216	77.59 ± 7.96	87.65 ± 12.77	9.7
LPS + zVAD	67.15 ± 3.56	88.59 ± 6.00	24.2[a]

This table compares the ability of two different NO-containing non-steroidal anti-inflammatory drugs and a pan caspase synthesis inhibitor, zVAD, to attenuate the effects of the chronic infusion of lipopolysaccharide (LPS) into the basal forebrain of young rats. Each data point represents the results, expressed as the mean ± S.E.M., obtained from six rats.

[a] $P < 0.05$ vs. either CSF, lipopolysaccharide + NO-flurbiprofen (NFP) or lipopolysaccharide + NCX2216.

regulate their expression of tissue antigens following inflammation (Finsen et al., 1993; Perry et al., 1993). To visualize activated microglia, a monoclonal antibody (OX-6, 1/400 dilution, PharMingen, San Diego, CA) directed against the Class II major histocompatibility complex (MHC II) was used (Flaris et al., 1993; Perry et al., 1993).

3. Results

All of the rats gained weight normally during the drug treatment period. None of the rats was observed to have seizure activity or demonstrate any other indication of toxicity or poor health.

3.1. Biochemical studies

Chronic infusion of lipopolysaccharide into the basal forebrain region for 37 days significantly decreased regional choline acetyltransferase activity on the side ipsilateral to the infusion (see Table 1), as compared to the un-injected (right) side of the brain [$F(4, 29) = 6.08$, $P = 0.0014$] or as compared to the CSF-infused rats [$F(4, 29) = 11.1$, $P = 0.0001$]. Choline acetyltransferase activity was also significantly ($P < 0.05$) decreased within the basal forebrain of rats infused with lipopolysaccharide that

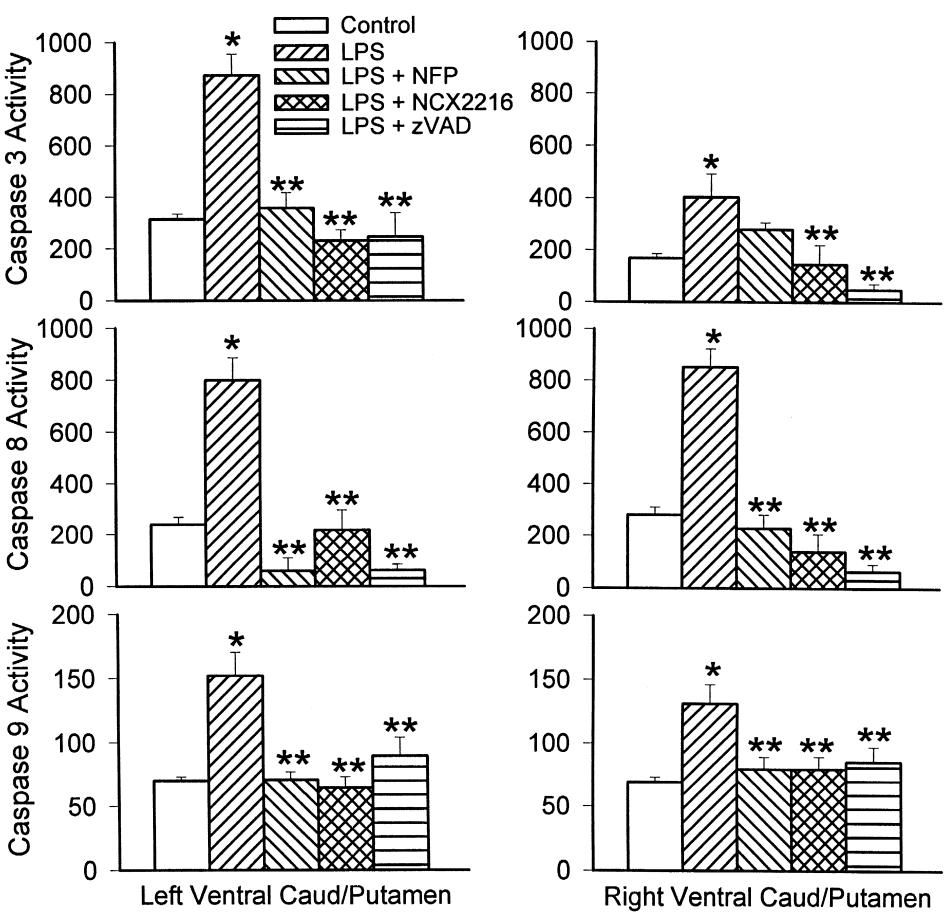

Fig. 1. Endogenous levels of caspases 3, 8 and 9 activity within the ventral caudate/putamen. Values for caspase activity are in optical density/mg protein and are expressed as mean ± S.E.M. [a] $P < 0.05$ vs. CSF, [b] $P < 0.05$ vs. lipopolysaccharide (LPS). Infusions of lipopolysaccharide were made into the left basal forebrain region.

were also co-infused with zVAD. Therefore, the co-infusion of a caspase synthesis inhibitor, zVAD, did not provide neuroprotection from the cytotoxic effects of lipopolysaccharide upon cholinergic cells. The level of choline acetyltransferase activity within the basal forebrain of rats infused with CSF did not differ significantly from the level of choline acetyltransferase activity in the basal forebrain of rats infused with either CSF or lipopolysaccharide-infused rats who were also treated chronically with either NO-flurbiprofen or NCX2216. Therefore, significant neuroprotection from the toxic effects of lipopolysaccharide upon cholinergic cells in the basal forebrain was provided by chronic administration of either NO-non-steroidal anti-inflammatory drug. Finally, the level of choline acetyltransferase activity within the right basal forebrain region, i.e. the un-injected side of the brain, did not differ significantly [$F(4, 29) = 1.36$, $P = 0.29$] between groups.

The level of caspase 3 within the ventral region of the caudate/putamen was significantly [both sides $F(4,29) > 4.5$, $P < 0.005$] increased on the left and right side of the brain by the lipopolysaccharide infusion, as compared to CSF-infused controls (see Fig. 1). In addition, the level of caspase 3 was significantly ($P < 0.05$) greater on the side of the brain that received the lipopolysaccharide infusion, as compared to the un-injected side. The infusion of aCSF alone significantly increased ($P < 0.05$) the level of caspase 3 on both sides of the brain; in addition, the level of caspase 3 was significantly ($P < 0.05$) greater on the left (injected) side of the brain, as compared to the right (un-injected) side of the brain. On the left side of the brain, the level of caspase 3 was significantly decreased ($P < 0.05$) by chronic treatment with both NO-non-steroidal anti-inflammatory drugs or the pan-caspase synthesis inhibitor zVAD, as compared to the effects of lipopoly-

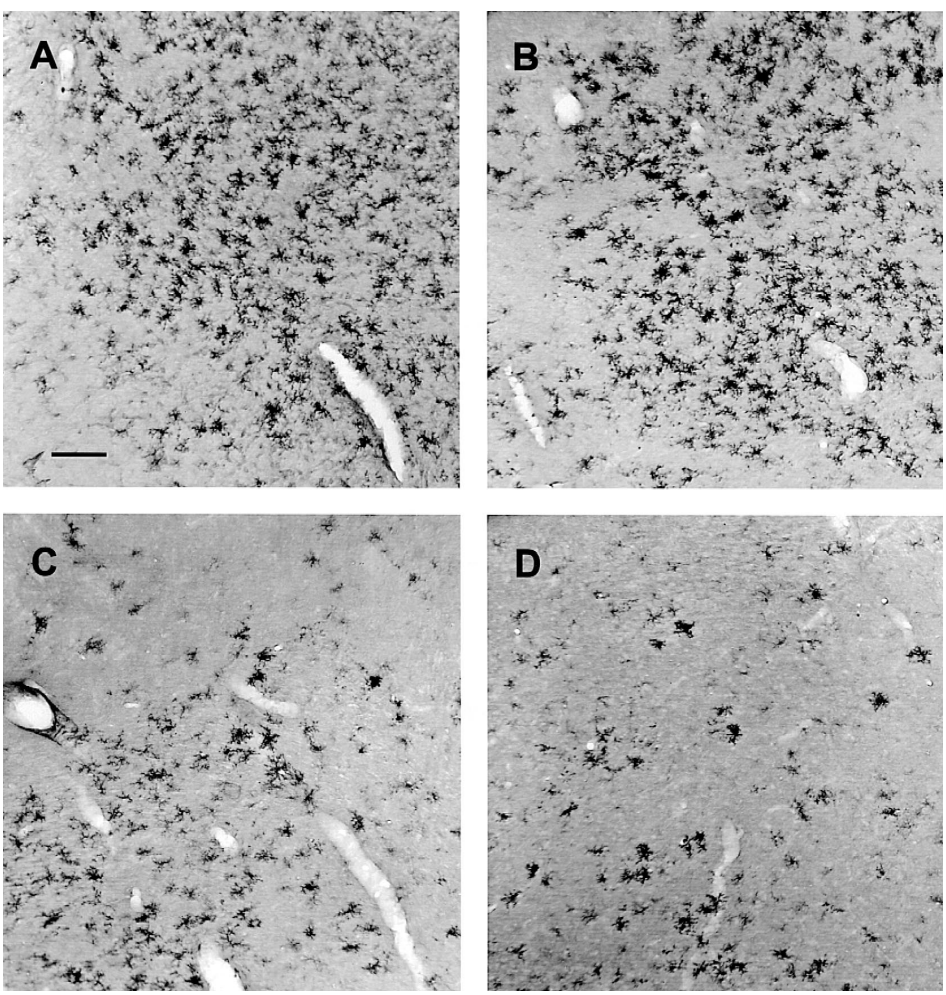

Fig. 2. Immunohistochemical staining for MHC II (using the OX6 antibody) within the basal forebrain region. All photographs were taken in an area ventral to the anterior commissure and within the substantia innominata. A — Lipopolysaccharide-infused rats. Activated microglia were distributed throughout the basal forebrain region. The reactive microglia in the lipopolysaccharide-treated rats were characterized by a contraction of their highly ramified processes that appeared bushy in morphology. B — Lipopolysaccharide-infused rats treated daily with zVAD. C — Lipopolysaccharide-infused rats treated daily with NO-flurbiprofen. D — Lipopolysaccharide-infused rats treated daily with NCX 2216. Bar = 65 μM

saccharide. In contrast, on the right side of the brain, the level of caspase 3 was significantly decreased ($P < 0.05$) by chronic treatment with only NCX2216 and the pan-caspase synthesis inhibitor zVAD, as compared to the effects of lipopolysaccharide.

The level of caspase 8 within the ventral region of the caudate/putamen was significantly [both sides $F(4, 29) > 21.9$, $P < 0.0001$] increased on both the left and right side of the brain by the lipopolysaccharide infusion, as compared to CSF-infused controls. The level of caspase 8 was not significantly ($P > 0.1$) greater on the side of the brain that received the lipopolysaccharide infusion, as compared to the un-injected side. The infusion of aCSF alone significantly increased ($P < 0.05$) the level of caspase 8 on both sides of the brain to the same degree. The level of caspase 8 was significantly decreased ($P < 0.05$) on both sides by chronic treatment with both NO-non-steroidal anti-inflammatory drugs or the pan-caspase synthesis inhibitor zVAD, as compared to the effects of lipopolysaccharide.

The level of caspase 9 within the ventral region of the caudate/putamen was significantly [both sides $F(4, 29) > 4.7$, $P < 0.005$] increased on the left and right side of the brain by the lipopolysaccharide infusion, as compared to CSF-infused controls. The level of caspase 9 was not significantly ($P > 0.1$) greater on the side of the brain that received the lipopolysaccharide infusion, as compared to the un-injected side. In addition, the infusion of aCSF alone significantly increased ($P < 0.05$) the level of caspase 9 on both sides of the brain to the same degree. The level of caspase 9 was significantly decreased ($P < 0.05$) on both sides by chronic treatment with both NO-non-steroidal anti-inflammatory drugs or the pan-caspase synthesis inhibitor zVAD, as compared to the effects of lipopolysaccharide.

3.2. Histological studies

Immunocytochemical staining for the MHC II (OX-6 antibody) revealed that the microglia within the basal forebrain developed a strong inflammatory response following chronic exposure to lipopolysaccharide (Fig. 2). In the basal forebrain of lipopolysaccharide-infused rats, the activated microglia were characterized by a contraction of their highly ramified processes giving them a bushy morphology (Fig. 2A). The basal forebrain of lipopolysaccharide-infused rats that were also co-infused with zVAD (Fig. 2B) did not appear to have fewer or less reactive microglia, as compared to the lipopolysaccharide-infused rats. Highly activated microglia were also distributed throughout the entire coronal section of the brain with the concentration of activated microglia greatest near the infusion site. Rats treated with aCSF had only a few reactive microglia (not shown, see Hauss-Wegrzyniak et al., 1999). Lipopolysaccharide-infused rats that were treated daily with either NO-flurbiprofen (Fig. 2C) or NCX 2216 (Fig. 2D) had fewer and somewhat less reactive microglia.

4. Discussion

In the present study, infusion of lipopolysaccharide into the basal forebrain of young rats produced extensive inflammation and a significant loss of cholinergic cells. These results are consistent with our previous findings, i.e. both acute and chronic infusions of lipopolysaccharide destroyed cholinergic cells and activated astrocytes and microglia (Wenk and Willard, 1999; Willard et al., 1999, 2000). In addition, the current study extended our understanding of the mechanisms by which chronic neuroinflammation destroys basal forebrain cholinergic cells. Inflammatory processes, such as exposure to lipopolysaccharide, that activate glia can lead to the release of cytokines and initiate a cascade of reactive oxygen intermediates (Goossens et al., 1995; Minghetti and Levi, 1995) and inflammatory proteins which can be detrimental to cellular function (Bluthe et al., 1992; Quan et al., 1994). During inflammatory processes in the brain that are associated with disease, a marked rise in the synthesis of prostaglandins is seen (Dubois et al., 1998). Increased prostaglandin production has been implicated in other dementia states that are associated with neuroinflammation (Griffin et al., 1994). The present study determined that peripheral administration of prostaglandin synthesis inhibitors, e.g. the NO-non-steroidal anti-inflammatory drugs, significantly attenuated the cytotoxic consequences of chronic neuroinflammation upon basal forebrain cholinergic cells. Chronic treatment with these drugs also attenuated the glial response to the neuroinflammation produced by lipopolysaccharide. These findings are also consistent with our previous reports (Hauss-Wegrzyniak et al., 1998; Wenk et al., 2000). We have also previously shown that long-term treatment with these NO-non-steroidal anti-inflammatory drugs alone does not influence brain function (Hauss-Wegrzyniak et al., 1998, 1999).

A fundamental question that has challenged previous hypotheses of neurodegeneration is why specific neuronal populations are affected more than others. Taken together with our previous findings (Willard et al., 2000), the results of the present study are consistent with the hypothesis that basal forebrain cholinergic neurons are vulnerable to the consequences of elevated levels of cytokines and prostaglandins produced by the condition of chronic neuroinflammation that characterizes the brain of Alzheimer's disease patients (Akiyama et al., 2000). Although the mechanism of this toxicity is unknown, we have recently demonstrated an important role for NMDA-sensitive glutamate receptors (Willard et al., 2000). We have hypothesized the following cascade of events in the degeneration of basal forebrain cholinergic cells (Wenk and Willard, 1999). Lipopolysaccharide infusions stimulate the endogenous production of inflammatory cytokines by activated astrocytes and microglia (Bluthe et al., 1992; Quan et al., 1994). Cytokines can in turn stimulate the production of a variety of other inflammatory mediators such as prosta-

glandins (Katsuura et al., 1989; Oka and Arita, 1991). Prostaglandins can induce the release of glutamate from astrocytes (Bezzi et al., 1998) leading to the stimulation of glutamate receptors, the depolarization-dependent unblocking of NMDA receptors by Mg^{2+}, and the entry of toxic amounts of Ca^{2+} into neurons. Prostaglandins and various cytokines may also indirectly elevate the extracellular concentration of glutamate by inhibiting its reuptake by astrocytes (Fine et al., 1996; Robinson et al., 1993; Rothstein et al., 1993). Previous studies have found that elevated levels of inflammatory proteins may selectively target cholinergic basal forebrain neurons (McMillian et al., 1995; Willard et al., 1999, 2000). Our recent findings (Wenk and Willard, 1999) suggest that the selective vulnerability of cholinergic neurons within the basal forebrain may be related to the fact that they receive a dense glutamate projection from the pedunculopontine tegmentum (Rasmusson et al., 1994) and are sensitive to excess stimulation of glutamate NMDA receptors (Wenk et al., 1995).

The lipopolysaccharide infusion elevated caspase levels on both sides of the basal forebrain but was associated with cholinergic cell loss only on the injected side of the brain. In addition, the treatment with zVAD significantly reduced endogenous caspase levels. This confirmed that the dose of zVAD chosen was sufficient to produce the expected effect upon caspase levels. However, this level of caspase synthesis inhibition, when produced for 37 continuous days in the presence of lipopolysaccharide, did not provide neuroprotection for cholinergic cells. Therefore, the current study found no correlation between endogenous levels of caspase produced in response to neuroinflammation and the degeneration of basal forebrain cholinergic neurons.

Finally, we hypothesize that this model of inflammation-induced lesions of the basal forebrain cholinergic neurons mimics the human condition associated with chronic glial activation and may thereby replicate the initial steps of the neurotoxic cascade that may lead to cell death in the Alzheimer's disease brain. Taken together with the results of previous studies (Hauss-Wegrzyniak et al., 1998, 1999; Gahtan and Overmier, 1999; McGeer and McGeer, 1998; Wenk et al., 2000; Wenk and Willard, 1999; Willard et al., 1999), the following scenario can be suggested that depends upon the ultimate role of glutamate as endogenous neurotoxin acting within the basal forebrain. The interaction of microglia within senile plaques containing beta-amyloid protein results in a chronic activation of these cells and the release of various cytokines and complement proteins. Elevated levels of cytokines and beta-amyloid proteins may then advance glutamate excitotoxicity within the Alzheimer's disease brain (Abbas et al., 1994) by releasing glutamate to over-stimulate NMDA receptors on glia as well as impairing glutamate uptake mechanisms and detoxification processes vital to neuronal survival (Aisen and Davis, 1994). Stimulated glia would also release cytokines that would then potentiate the toxicity of glutamate (Dommergues et al., 2000). The long-term exposure of cholinergic cells to elevated levels of extracellular glutamate may ultimately lead to their gradual degeneration as the behavioral symptoms progress, particularly during the advanced stages of the disease (Davis et al., 1999). The results of the present study suggest that chronic combination therapy with an NO-non-steroidal anti-inflammatory drug and a NMDA receptor channel antagonist, e.g. memantine (Wenk and Willard, 1999; Willard et al., 2000) might attenuate the loss of forebrain cholinergic neurons and delay the onset of the cognitive impairments associated with their degeneration.

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