Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Rapid Communication

Intracerebral NMDA Injection Stimulates Production of Interleukin-1β in Perinatal Rat Brain

*P. Hagan, †S. Poole, †A. F. Bristow, ‡F. Tilders, and *§F. S. Silverstein

*Departments of *Pediatrics and §Neurology, University of Michigan, Ann Arbor, Michigan, U.S.A.; †Department of Endocrinology, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, England; and ‡Department of Pharmacology, Free University of Amsterdam, Amsterdam, The Netherlands*

Abstract: Susceptibility to NMDA neurotoxicity peaks in the early postnatal period in rats. Although indirect evidence suggests that interleukin-1β is a mediator of NMDA neurotoxicity in perinatal rats, direct confirmation of NMDA-induced interleukin-1β production in the brain has not been reported previously. The primary goal of this study was to determine if intracerebral injection of a neurotoxic dose of NMDA stimulates interleukin-1β production acutely. We used a rat-specific interleukin-1β ELISA to quantify brain tissue homogenate interleukin-1β content, and an immunocytochemical assay with a monoclonal anti-rat interleukin-1β antibody to visualize its distribution. NMDA (10 nmol) was injected stereotaxically into 7-day-old rats, using coordinates that targeted the striatum and overlying dorsal hippocampus. Interleukin-1β concentrations were measured in samples from the injected and contralateral cerebral hemispheres 0–12 h later; in addition, the impact of treatment with the noncompetitive NMDA antagonist MK-801 on interleukin-1β production was assessed. We found marked increases in tissue content of interleukin-1β in the lesioned hemisphere; values peaked at 6 h post injection. Treatment with MK-801 (1 mg/kg) blocked NMDA-induced increases in interleukin-1β. Preliminary immunocytochemical analysis demonstrated high concentrations of interleukin-1β-immunoreactive cells in the lesioned hippocampus, and concurrent increases in interleukin-1β immunoreactivity diffusely in the ependyma at 6 h after NMDA administration. Our data provide the first direct evidence that NMDA-induced excitotoxic injury stimulates interleukin-1β production in vivo. **Key Words:** Interleukin-1β—*N*-Methyl-D-aspartate—MK-801—Rat brain.
J. Neurochem. **67,** 2215–2218 (1996).

Interleukin-1β (IL-1β) is a pleiotropic cytokine that plays important roles in immune, endocrine, and inflammatory responses (Dinarello, 1994). In the CNS, recent experimental evidence implicates IL-1β as a mediator of neuronal damage caused by ischemic and excitotoxic brain injury. Transient increases in IL-1β mRNA expression after ischemic, traumatic, and excitotoxic injury have been reported (Minami et al., 1990, 1992; Fan et al., 1995; Szaflarski et al., 1995). Although increased IL-1β bioactivity was detected following mechanical injury (Woodroofe et al., 1991), direct evidence that acute brain injury stimulates mature IL-1β production in the brain has not been reported previously.

The hypothesis that IL-1β contributes to the progression of acute brain injury stems from findings that pharmacological antagonism of IL-1β by intraventricular infusion of IL-1 receptor antagonist (IL-1ra) (a specific receptor antagonist that blocks many biological actions of IL-1β) reduced both excitotoxic and ischemic damage in adult rat brain (Relton and Rothwell, 1992), and from evidence that adenoviral vector-mediated overexpression of IL-1ra in the brain attenuated ischemic (Betz et al., 1995) and excitotoxic (Hagan et al., 1996) injury.

Intracerebral NMDA injection in postnatal day 7 (P7) rats elicits reproducible, dose-dependent neuronal damage; susceptibility to NMDA neurotoxicity peaks at this age (McDonald et al., 1988). Our findings that intracerebral NMDA injection stimulated IL-1β mRNA expression (Szaflarski et al., 1995) and that adenovirus-mediated overexpression of IL-1ra conferred resistance to NMDA-induced brain injury (Hagan et al., 1996) prompted us to evaluate whether increased IL-1β could be detected acutely in lesioned brain, and whether its expression corresponded with the distribution of neuronal injury. IL-1β protein was quantified using a rat IL-1β ELISA; preliminary experiments were also performed to evaluate its neuroanatomic distribution immunocytochemically, by using a monoclonal rat-specific IL-1β antibody. Our results demonstrate the temporal and anatomic distribution of NMDA-induced IL-1β production in P7 rat brain.

MATERIALS AND METHODS

Previously reported methods were used for NMDA injections (McDonald et al., 1989). Surgical protocols were approved by the University of Michigan Committee on Care and Use of Animals in Research. P7 rats were anesthetized by methoxyfluorane inhalation, and NMDA [10 nmol/0.5

Resubmitted manuscript received August 12, 1996; accepted August 13, 1996.

Address correspondence and reprint requests to Dr. F. S. Silverstein at University of Michigan, Room 8301, MSRB 3, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0646, U.S.A.

Abbreviations used: IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; OD, optical density; P, postnatal day; PBS, phosphate-buffered saline; RT, room temperature; WDB, wash dilution buffer.

μl of phosphate-buffered saline (PBS), pH 7.4] was stereotaxically injected into the right striatum (coordinates relative to bregma: AP: 0 mm; L: 2.3 mm; D: 4.0 mm). This lesioning method, which targets both the striatum and adjacent structures, elicits substantial ipsilateral tissue damage; 5 days later, there is marked neuronal necrosis and atrophy in the striatum, adjacent dorsal hippocampus (particularly in the CA3 subfield), and overlying cortex and a 10% reduction in the weight of the lesioned cerebral hemisphere compared with the contralateral side (McDonald et al., 1989).

In preliminary experiments, to minimize tissue handling, the brains were rapidly removed, the hemispheres were separated on ice, and each hemisphere was homogenized in 0.5 ml of PBS (4°C) in <1 min; samples were centrifuged (10,000 rpm, 10 min, 4°C), and the supernatant was stored at −70°C. Values in the range of the IL-1 ELISA (see below) were obtained from a single hemisphere; therefore, this sampling method, which did not require tissue pooling, was selected.

In the first group of experiments, animals received intrastriatal NMDA injections (10 nmol) and were killed by decapitation 0, 2, 4, 6, or 12 h later (n = 4 per group, except at time 0, n = 2); four controls received intrastriatal injections of 0.5 μl of PBS, pH 7.4, and were killed 12 h later. Samples were prepared from the left and right cerebral hemispheres of each animal.

In the next experiment, to evaluate the effect of treatment with (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), a noncompetitive NMDA receptor antagonist, on IL-1β production, animals were treated with intraperitoneal injections of MK-801 (1 mg/kg in 0.1 ml) 15 min after NMDA lesioning or with an equal volume of saline (n = 4 per group). An additional control group (n = 3) received intrastriatal saline injections (0.5 μl), followed 15 min later by an intraperitoneal injection of the same dose of MK-801. This MK-801 dose blocks NMDA-induced injury (McDonald et al., 1989). All animals were killed 6 h later, and tissue samples were prepared as above. Samples from normal P7 animals (n = 4) were assayed in two subsequent experiments.

Four additional animals received stereotaxic intracerebral NMDA injections; 6 h later, animals received lethal overdoses of chloral hydrate (400 mg/kg) and were perfused with 2% paraformaldehyde/PBS, pH 7.4. The brains were removed and cryoprotected. Twenty-micrometer frozen sections were used immediately for immunocytochemistry. In preliminary experiments we found that more prolonged storage of either whole brains or frozen sections resulted in marked loss of IL-1β immunoreactivity.

IL-1β content of tissue homogenates was measured using a rat IL-1β ELISA (Garabedian et al., 1995). Ninety-six-well immunoplates were coated for 16 h with immunoaffinity-purified polyclonal sheep anti-rat IL-1β (4 μg/ml in PBS, pH 7.2, 100 μl/well) and washed three times in a high-salt wash dilution buffer [WDB; 0.5 *M* NaCl, 2.5 m*M* NaH_2PO_4, 7.5 m*M* Na_2HPO_4, 0.1% (vol/vol) Tween 20]; then triplicate samples and standards (100 μl/well recombinant rat IL-1β) were added and incubated for 16 h at 4°C. Plates were washed in WDB, incubated with biotinylated affinity-purified polyclonal sheep anti-rat IL-1β [1:1,000 dilution in WDB containing 1% normal sheep serum, 100 μl/well, 1 h at room temperature (RT)], washed three times, and incubated with avidin-horseradish peroxidase (1:5,000, 15 min at RT). After three washes in WDB, samples were incubated with *o*-phenylenediamine (0.2 mg/ml, 100 μl/well, 30 min at RT), and the reaction was terminated with 1 *M* H_2SO_4 (150 μl/well). An automated plate reader was used to measure optical density (OD) at 490 nm, and IL-1β content was derived from a standard graph. Data were analyzed by ANOVA, and intergroup differences were evaluated with post-hoc *t* tests.

Assay reagents were prepared by Drs. Poole and Bristow; recombinant rat IL-1β was generously provided by Dr. R. Newton (Du Pont–Merck). This ELISA has a sensitivity of 8 pg/ml (cytokine concentrations that yield OD values ≥2 SD above those obtained for 0 pg/ml cytokine); the rectilinear relation between log concentration and log OD extends up to 1,000 pg/ml. There is cross-reactivity of mouse and human IL-1β in this ELISA (dose–response curves lie two logs to the right); mouse IL-1α at concentrations above 30 ng/ml show minimal cross-reactivity (curve lies five logs to the right).

The immunocytochemical assay of rat IL-1β was developed using a monoclonal mouse anti-rat IL-1β antibody (SILK20; Schotanus et al., 1995) (3 μg/ml, 1:1,500 dilution). This antibody recognizes the amino acid sequence 78–98 of mature rat IL-1β and binds with high affinity (7 nmol/L) to recombinant rat IL-1β. Slide-mounted 20-μm sections (≥20 per brain) were postfixed for 10 min at RT in 2% paraformaldehyde/PBS, pH 7.4, washed three times with buffer 1 (PBS/0.4% Triton X-100/1% normal horse serum), incubated with a nonspecific blocking serum (10% normal horse serum diluted in buffer 1; 20 min at RT), and incubated for 16 h with the primary antibody. Sections were washed three times in buffer 1 and incubated with the secondary antibody (biotinylated horse anti-mouse, 1:500 dilution in buffer 1, 1 h, RT). After three PBS rinses, sections were incubated with avidin complex, biotinylated enzyme, and chromogen (ABC Elite kit, Vector, Burlingham, CA, U.S.A.). Control sections in which the primary antibody was replaced with an equivalent dilution of species-matched IgG were assayed concurrently, and yielded no staining. Sections were counterstained with cresyl violet.

RESULTS

NMDA (10 nmol) stimulated IL-1β production in the lesioned cerebral hemisphere in the first 12 h after lesioning (Fig. 1; $p < 0.001$, ANOVA, comparing changes over time and side–side differences). Increased production in the right hemisphere was measured as early as 2 h postinjection (threefold increase over contralateral hemisphere concentrations), and peak concentrations (572 ± 85 pg/ml, ipsilateral hemisphere, mean ± SEM) were detected at 6 h post lesioning. At 12 h, IL-1β concentrations in the right hemisphere remained elevated to a lesser degree. No significant change in IL-1β production was detected in the contralateral hemisphere.

Treatment with a neuroprotective dose of MK-801 blocked NMDA-induced IL-1β production (Fig. 2), measured at 6 h post lesioning ($p < 0.001$). MK-801 had no suppressive effect on IL-1β production in controls that had received intrastriatal saline injections. In this experiment, in the NMDA-lesioned, intraperitoneal saline-treated group, concentrations of IL-1β in samples from the right hemisphere were in the same range as peak concentrations attained in the preceding experiments (620 ± 28 vs. 572 ± 85 pg/ml, mean ± SEM). However, in all three experimental groups, IL-1β values in samples from the contralateral hemisphere were somewhat higher than those observed earlier; factors that account for this difference are uncertain. In two subsequent independent assays of normal P7 rat brain, the mean IL-1β value was 180 pg/ml, similar to the contralateral hemisphere values in this experiment.

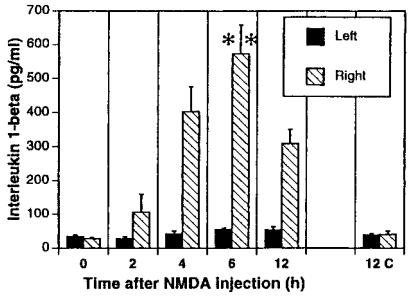

FIG. 1. NMDA-induced IL-1β production. P7 rats received right intracerebral injections of NMDA (10 nmol/0.5 μl); tissue IL-1β content was measured 0–12 h later. Brain homogenates were prepared from each cerebral hemisphere; an ELISA was used to measure IL-1β (see Materials and Methods), and absolute values were derived from internal standards. Data, expressed as mean ± SEM (bars) values (n = 4 per group, except at time 0, where n = 2), represent concentrations in tissue homogenates. "Right" and "Left" identify injected and contralateral cerebral hemisphere values, respectively. "12C" represents values from controls that received intrastriatal saline injections and were killed 12 h later. $^{**}p < 0.001$ (two-way ANOVA, comparing side–side differences and changes over time).

Our preliminary immunocytochemical studies demonstrated that NMDA induced IL-1β immunoreactivity in the lesioned hemisphere at 6 h post lesioning (Fig. 3). Increased IL-1β immunoreactivity was detected most readily within the pyramidal cell layer of the CA3 subfield of the lesioned hippocampus (Fig. 3B), the region most susceptible to NMDA neurotoxicity in P7 rats. IL-1β-immunoreactive cells were also diffusely distributed in the adjacent lesioned striatum (results not shown). High concentrations of immunoreactive cells were also detected in the ependyma, both ipsilateral and, to a lesser degree, contralateral to the lesion. In three additional brains (results not shown), the findings were the same; the pattern of immunoreactivity was similar in all sections that were assayed.

DISCUSSION

Our data demonstrate that NMDA stimulates production of IL-1β in vivo in neonatal rat brain, and that treatment

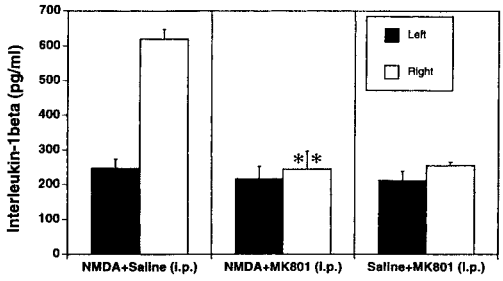

FIG. 2. MK-801 inhibits NMDA-induced IL-1β production. P7 rats received right intracerebral injections of NMDA (10 nmol/0.5 μl; n = 4 per group) or saline (n = 3); 15 min later they received injections (0.1 ml i.p.) of MK-801 (1 mg/kg) or an equal volume of saline. Tissue IL-1β content was evaluated at 6 h post lesioning. An ELISA was used to measure IL-1β in brain homogenates (see Materials and Methods). Absolute values, derived from internal standards, were expressed as mean ± SEM (bars) values; "Right" and "Left" identify injected and contralateral cerebral hemisphere values, respectively. $^{**}p < 0.001$ (ANOVA, with post-hoc comparison of right hemisphere values in MK-801- and saline-treated NMDA-lesioned groups).

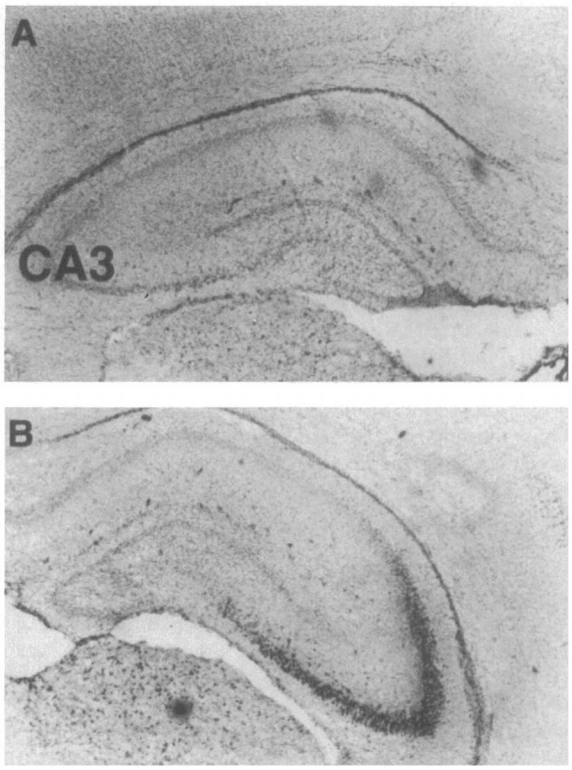

FIG. 3. NMDA-induced IL-1β expression. This montage demonstrates the distribution of IL-1β-immunoreactive cells in the left (**A**) and right (**B**) hippocampus of a P7 animal that had received a right intracerebral injection of NMDA (10 nmol/0.5 μl) 6 h earlier. A monoclonal mouse anti-rat IL-1β antibody was used to detect IL-1β (see Materials and Methods); immunoreactive cells were visualized with 3,3′-diaminobenzidine, which yielded a dark precipitate, and sections were counterstained with cresyl violet to identify anatomical landmarks. In A, there is subtle evidence of increased immunoreactivity, limited to the ependyma. In contrast, in the lesioned hippocampus (B), immunoreactive cells are concentrated in the CA3, the region most susceptible to NMDA neurotoxicity at this developmental stage. Control sections (not shown), in which an equal concentration of isotype-matched IgG was substituted for the primary antibody, had no reactive cells. CA, cornu ammonis. Magnification, A and B: ×4.

with the noncompetitive NMDA antagonist MK-801 blocks this response. The timing of peak protein expression (6 h post injection) is congruent with the temporal pattern of IL-1 mRNA expression, peaking at 4 h after lesioning (Szaflarski et al., 1995).

In contrast with the findings that ischemic, excitotoxic, and traumatic brain injury transiently stimulated IL-1β mRNA expression (Minami et al., 1990, 1992; Fan et al., 1995; Szaflarski et al., 1995); direct evidence that acute brain injury stimulated production of mature IL-1β protein has been lacking. Our data suggest that technical factors, related to methods of sample preparation and/or protein detection, may account for this disparity. Moreover, our data are congruent with two studies of IL-1 functional activity in P7 rat brain. Giulian et al. (1988) first reported that IL-1 bioactivity could be detected in normal P7 (but not in adult) rat brain, a finding that is consistent with our immunological data. Of particular relevance, in a recent study of IL-1 bioactivity in P7 rat brain after hypoxic–ischemic lesioning,

Hagberg et al. (1995) demonstrated peak activity in the injured cerebral hemisphere at 6 h after lesioning, a temporal pattern that coincided closely with our results.

IL-1β could exert either beneficial or deleterious effects after acute brain injury (Strijbos and Rothwell, 1995). Yet in vivo, in the setting of acute brain injury at the developmental stage used in our study, pharmacological antagonism of IL-1β is clearly neuroprotective (Martin et al., 1994; Hagan et al., 1996).

Whether IL-1β mediates NMDA-induced excitotoxic damage directly or indirectly is uncertain. IL-1β regulates expression of a host of other cytokines and potential neurotoxic mediators. For example, IL-1β potently stimulates inducible nitric oxide synthase activity; in vitro increased inducible nitric oxide synthase activity potentiates NMDA neurotoxicity (Hewett et al., 1994).

Our preliminary immunocytochemistry data suggest that injured neurons could be a major source of IL-1β production, as were ependymal cells. Surprisingly few immunoreactive cells with microglial morphology (Van Dam et al., 1992) were seen. To evaluate whether the immunoreactive cells within the pyramidal cell layer could be microglia or monocytes, we performed preliminary lectin histochemistry assays to identify these cells in NMDA-lesioned brain (Ivacko et al., 1996); at 6 h after lesioning, although we could detect subtle evidence of microglial activation ipsilaterally, very few lectin-stained cells infiltrated the pyramidal cell layer, where IL-1β immunoreactivity was concentrated (unpublished observations). As IL-1β is secreted, an alternative interpretation of the distribution of immunoreactivity would be that it reflects regions where secreted IL-1β bound to cell surface receptors.

Overactivation of NMDA receptors initiates a complex cascade of interrelated intracellular events that lead to irreversible injury. In clinical disorders mediated by NMDA receptor overactivation, injury evolves progressively; if inflammatory mediators, such as IL-1β, produced several hours after the onset of injury are neurotoxic, then therapeutic interventions targeted to block their deleterious actions, administered after the initial CNS insult, could be neuroprotective. A greater understanding of the complex role(s) of IL-1β and related proinflammatory cytokines in the pathogenesis of acute brain injury is an essential prerequisite for the development of effective neuroprotective therapies based on pharmacological antagonism of IL-1β.

Acknowledgment: This work was supported by grants from the Hearst Foundation, the United Cerebral Palsy Education and Research Foundation, and the U.S.P.H.S. (NS 31054) to F.S.S. Dr. John Barks provided valuable assistance. The IL-1β standard was generated at Du Pont–Merck and kindly provided to S.P. by Dr. Robert Newton. An abstract of these data was submitted to the 1996 meeting of the Society for Neuroscience (Washington, D.C.).

REFERENCES

Betz A. L., Yang G.-Y., and Davidson B. L. (1995) Attenuation of stroke size in rats using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist in brain. *J. Cereb. Blood Flow Metab.* **15,** 547–551.

DaCunha A., Jefferson J. J., Tyor W. R., Glass J. D., Jannotta F. S., and Vitkovic L. (1993) Control of astrocytosis by interleukin-1 and transforming growth factor-beta in human brain. *Brain Res.* **631,** 39–45.

Dinarello C. A. (1994) The interleukin-1 family: 10 years of discovery. *FASEB J.* **8,** 1314–1322.

Fan L., Young P. R., Barone F. C., Feuerstein G. Z., Smith D. H., and McIntosh T. K. (1995) Experimental brain injury induces expression of interleukin-1b mRNA in the rat brain. *Mol. Brain Res.* **30,** 125–130.

Garabedian B. S., Poole S., Allchorne A., Winter J., and Woolf C. J. (1995) Interleukin-1β contributes to the inflammation-induced increase in nerve-growth factor levels and inflammatory hyperalgesia. *Br. J. Pharmacol.* **115,** 1265–1275.

Giulian D., Young D. G., Woodward J., Brown D. C., and Lachman L. B. (1988) Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci.* **8,** 709–714.

Hagan P., Barks J. D. E., Yabut M., Davidson B., Roessler B., and Silverstein F. S. (1996) Adenovirus-mediated over-expression of interleukin-1 receptor antagonist reduces susceptibility to excitotoxic brain injury in perinatal rats. *Neuroscience* (in press).

Hagberg H., Soder O., Gilland E., McRae A., Bona E., and Blennow M. (1995) Interleukin-1 and hypoxic–ischemic brain injury in the neonatal rat. *Pediatr. Res.* **37,** 380A.

Hewett S. J., Cseranky C. A., and Choi D. W. (1994) Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron* **13,** 487–494.

Ivacko J. A., Sun R., and Silverstein F. S. (1996) Hypoxic–ischemic brain injury induces an acute microglial reaction in perinatal rats. *Pediatr. Res.* **39,** 39–47.

Martin D., Chinookoswong F. N., and Miller G. (1994) The interleukin-1 receptor antagonist (rhIL-1ra) protects against cerebral infarction in a rat model of hypoxia–ischemia. *Exp. Neurol.* **130,** 362–367.

McDonald J. W., Silverstein F. S., and Johnston M. V. (1988) Neurotoxicity of NMDA is markedly enhanced in developing rat central nervous system. *Brain Res.* **459,** 200–203.

McDonald J. W., Roeser N. F., Silverstein F. S., and Johnston M. V. (1989) Quantitative assessment of neuroprotection against NMDA-induced brain injury. *Exp. Neurol.* **106,** 289–296.

Minami M., Kuraishi Y., Yamaguchi T., Nakai S., Hirai Y., and Satoh M. (1990) Convulsants induce interleukin-1beta mRNA in rat brain. *Biochem. Biophys. Res. Commun.* **171,** 832–837.

Minami M., Kuraishi Y., Yabuuchi K., Yamazaki A., and Satoh M. (1992) Induction of interleukin-1β mRNA in rat brain after transient forebrain ischemia. *J. Neurochem.* **58,** 390–392.

Relton J. K. and Rothwell N. J. (1992) Interleukin-1 receptor antagonist inhibits ischemic and excitotoxic neuronal damage in the rat. *Brain Res. Bull.* **29,** 243–246.

Schotanus K., Meloen R. H., Puijk W. C., Berkenbosch F., Binnekade R., and Tilders F. J. (1995) Effects of monoclonal antibodies to specific epitopes of rat interleukin-1 beta (IL-1) on IL-1 beta-induced ACTH, corticosterone, and IL-6 responses in rats. *J. Neuroendocrinol.* **7,** 255–262.

Strijbos P. J. and Rothwell N. J. (1995) Interleukin-1 beta attenuates excitatory amino acid induced neurodegeneration in vitro: involvement of nerve growth factor. *J. Neurosci.* **15,** 3468–3474.

Szaflarski J., Burtrum D., and Silverstein F. S. (1995) Cerebral hypoxia–ischemia stimulates cytokine gene expression in perinatal rats. *Stroke* **26,** 1093–1100.

Van Dam A. M., Broune M., Louisse S., and Berkenbosch F. (1992) Appearance of interleukin-1 in macrophages and in ramified microglia in the brain of endotoxin-treated rats: a pathway for the induction of non-specific symptoms of sickness? *Brain Res.* **588,** 291–296.

Woodroofe M. N., Sarna G. S., Wadhwa M., Hayes G. M., Louglin A. J., Tinker A., and Cuzner M. L. (1991) Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. *J. Neuroimmunol.* **33,** 227–236.