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Inflammatory Regulators in Parkinson's Disease: iNOS, Lipocortin-1, and Cyclooxygenases-1 and -2

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Degeneration of dopaminergic neurons and focal gliosis are pathological hallmarks of Parkinson's disease and although the brain is described as immune-privileged focal immune reactions surround failing nigral neurons. We examined the cellular distribution of pro- and anti-inflammatory molecules in human parkinsonian and neurologically normal substantia nigra and caudate–putamen postmortem. An up-regulation of nitric oxide synthase- and cyclo-oxygenase-1- and -2-containing amoeboid microglia was found in parkinsonian but not control nigra. Astroglia contained low levels of these molecules in both groups. Lipocortin-1-immunoreactive amoeboid microglia were present within the astrocytic envelope of neurons adjacent to or within glial scars in parkinsonian nigra only. Lipocortin-1 is known to have neuroprotective and anti-inflammatory properties. Up-regulation of nitric oxide synthase is generally associated with neurodestruction whereas prostaglandin synthesis may be either neurodestructive or protective. The balance of these molecules is likely to be decisive in determining neuronal survival or demise.

INTRODUCTION

The primary pathology underlying Parkinson's disease is degeneration of pigmented dopaminergic neurons in the lateral and ventral substantia nigra pars compacta and focal gliosis which in severe cases of neuronal attrition form extensive nigral scars (Forno *et al.*, 1992; Forno, 1996; McGeer *et al.*, 1988; Knott *et al.*, 1999). Degenerating neurons, initially covered by astroglial processes, are surrounded by ramified microglia (Knott *et al.*, 1999). Subsequently, astroglial processes withdraw from degrading neuronal somata and amoeboid microglia accumulate within the astrocyte envelope (Knott *et al.*, 1999). This raises the possibility that astroglia and microglia communicate and that glial-derived cytokines might influence the fate of vulnerable dopaminergic neurons *in vivo.* In culture, media conditioned either by rodent astroglia (O'Malley *et al.*, 1991, 1992) or by microglia (Nagata *et al.*, 1993) support dopaminergic neuron survival. Neurotrophic factors are synthesized by astrocytes cultured from rodent "hemiparkinsonian"-lesioned brain (Langan *et al.*, 1995). *In vivo,* activated microglia express neurotrophic factors and induce dopaminergic sprouting in the injured rodent striatum (Batchelor *et al.*, 1999). In contrast, glia may also release cytokines with destructive capacity onto failing neurons (Vaca and Wendt, 1992; Giulian *et al.*, 1993; Banati *et al.*, 1993; Gehrmann *et al.*, 1995). Cultured microglia and astroglia produce large amounts of prostanoids, nitric oxide (NO), and proinflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), many of which are elevated following CNS insults (Giulian and Lachman, 1985; Sawada *et al.*, 1989; Giulian *et al.*, 1993). Microglia possess Fc receptors, CD4 antigen, and multiple histocompatibility complex class I and II antigens (McGeer *et al.*, 1988; Tooyama *et al.*, 1990) conferring them with antigen-presenting ability.

NO is synthesized from arginine by nitric oxide synthase (NOS; EC 1.14.13.39) of which there are three forms. Two are calcium- and calmodulin-dependent constitutive isoforms: eNOS (or type III) found in endothelia mediates arterial vasodilatation and vascular tone (Furchgott and Zawadzki, 1980) and cNOS (nNOS, bNOS, or type I) found in neurons acts as a neuromodulator by activating soluble guanylyl cyclase to induce

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cyclic GMP accumulation in the CNS (Garthwaite, 1991). Recent studies have indicated that, the term "constitutive" is no longer entirely appropriate for these forms of NOS since both forms can be induced by suitable stimuli. For example, both protein and catalytic activity of types 1 and 3 NOS are up-regulated following cerebral ischemia (Samdani *et al.*, 1997; O'Mahony and Kendall, 1999). Type 1 NOS is induced in the rodent stratum radiatum of CA1 and CA3 hippocampal subfields and dentate gyrus following perforant pathway stimulation (Lumme *et al.*, 2000), following kainate-induced status epilepticus in rodent cerebral cortex (Huh *et al.*, 2000), and in rodent cerebellar Purkinje cells by methyl mercury administration (Ikeda *et al.*, 1999) and it is down-regulated in rat forebrain slices following oxygen–glucose deprivation (De Alba *et al.*, 1999). Type 3 expression is up-regulated following cyclosporine A, via the "activator protein-1" *cis*-regulatory element (Navarro-Antolin *et al.*, 2000), by estrogen, a receptor-α-mediated effect (Tan *et al.*, 1999), and by lipopolysaccharide treatment in rat brain (Iwase *et al.*, 2000) and other stimuli such as hyperglycemia, the effects of which are thought to be mediated by hydrogen peroxide (Drummond *et al.*, 2000). A calcium-independent readily inducible form, iNOS (or type II), is found in astrocytes, microglia, macrophages, and neutrophils in culture (Lee *et al.*, 1993; Minc-Golomb and Schwartz, 1994; Chao *et al.*, 1996; Murphy, 2000) and is up-regulated following exposure to TNF-α, interferon-γ (IFNγ), and IL-1 (Moncada *et al.*, 1991; Chao *et al.*, 1996; Murphy and Grzybicki, 1996). TNF-α, IL-6, and IL-1β are elevated in the caudate–putamen and CSF in parkinsonian patients (Mogi *et al.*, 1994a,b; Müller *et al.*, 1988). TNF-α-immunoreactive (IR) glia (presumed to be microglia) were detected in the vicinity of degenerating nigral dopaminergic neurons bearing TNF receptors in Parkinson's diseased but not control nigra (Boka *et al.*, 1994). TNF-α presumably signals the up-regulation of glial iNOS reported in idiopathic Parkinson's disease (IPD; Hunot *et al.*, 1996). Although the exact cell sources of iNOS or TNF-α were not identified in these studies, glial-derived NO mediated neuron and oligodendrocyte death in culture (Boje and Arora, 1992; Merrill *et al.*, 1993) and might be involved in the pathology of IPD. TNF-α increased intracellular calcium currents in superior cervical ganglion cells in culture (Soliven and Albert, 1992) and might therefore raise intracellular calcium and consequently calcium-binding proteins, in IPD nigral neurons.

Lipocortin-1 (LC-1; annexin-1) is a calcium- and membrane-binding protein that buffers intracellular calcium, has voltage-gated calcium channel activity, promotes vesicle fusion and secretion, and has roles in cell proliferation and differentiation, coagulation, pyrogenesis, and inflammation (see Raynal and Pollard, 1994). LC-1 is present in CNS neurons (Strijbos *et al.*, 1991) and, despite lacking a signal sequence, is secreted from macrophages as part of the anti-inflammatory cascade (Solito *et al.*, 1993; Flower and Rothwell, 1994; Philip *et al.*, 1997). We have shown previously that it is secreted from reactive rodent microglia following kainate (KA)-mediated cerebellar damage (Hirst *et al.*, 1999) and that reactive astrocytes also produce LC-1 (Mullens *et al.*, 1994; Hirst *et al.*, 1999). LC-1 is neuroprotective when infused into damaged rat CNS (Relton *et al.*, 1991; Black *et al.*, 1992) and up-regulated in multiple-sclerosis-damaged CNS (Elderfield *et al.*, 1992). Although there is no gross inflammatory response in IPD nigra, localized inflammatory foci are likely (McGeer *et al.*, 1988; Kuhn and Muller, 1995). Since LC-1 down-regulates the release of reactive oxygen intermediates and proinflammatory eicosanoids in human monocytes (Sudlow *et al.*, 1996) if present in IPD nigra it might limit local inflammation.

Prostaglandins are synthesized from arachidonic acid (5,8,11,14-eicosatetraenoic acid) by cyclooxygenase (Cox) of two isoforms: Cox-1 (EC 1.14.99.1), a predominantly constitutive form involved in cellular homeostasis (O'Neill and Hutchinson, 1993), and Cox-2, an inducible isoform up-regulated by inflammatory cytokines, mitogens, and reactive oxygen intermediates (Williams and DuBois, 1996; Herschman, 1996; Smith and DeWitt, 1996). Inhibition of Cox *in vivo* protected against ischemic damage (Yamamoto *et al.*, 1995; Nogawa *et al.*, 1997), KA-induced seizures, and neurotoxicity in the rodent (Baran *et al.*, 1994). Cytotoxic effects of Cox are thought to be mediated through reactive oxygen species generated *within neurons themselves* by a peroxidase conversion of prostaglandin (PG)-G_2 to PGH_2 (Nogawa *et al.*, 1997). However, *prostaglandins can protect* cultured rat neurons against glutamate and hypoxic damage (Akaike *et al.*, 1994; Cazevielle *et al.*, 1994; Otsuki *et al.*, 1994).

Neuroprotective effects of prostaglandins may be mediated by glia. We and others found that reactive astrocytes express Cox-2 following damage (Hirst *et al.*, 1999; Sandhya *et al.*, 1998) and that astrocytes cultured from rodent cortex, cerebellum, and spinal cord synthesize and release PGD_2, PGE_2, prostacyclin, thromboxane A_2, and $PGF_{2\alpha}$ (Wilkin and Marriott, 1993). Others have found that PGE_2 down-regulated a number of microglial functions in culture including cytokine and NO production (Minghetti *et al.*, 1997, 1999b,c; Caggiano and Kraig, 1999). Microglia might therefore be an important target for astroglial prostaglandins *in vivo*

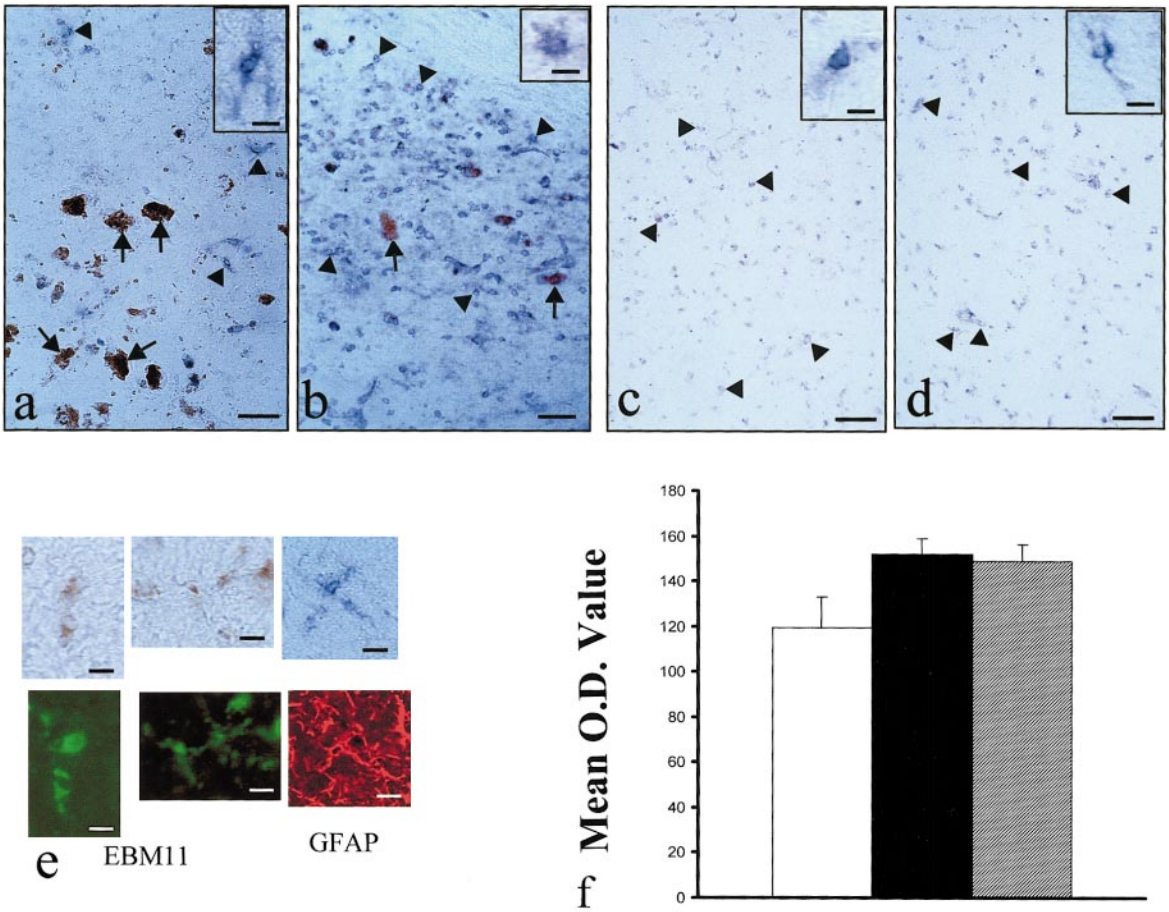


FIG. 1. Photomicrographs of iNOS-IR in control (a) and IPD (b) nigra and control (c) and IPD (d) putamen; bars, 45 μm. (Insets in a–d) Representative glia at higher magnification; bars, 15 μm. Glia in a–d are indicated by arrowheads and pigmented neurons in a and b are arrowed. (e) Dual labeling of representative iNOS-IR glia from IPD sections (light micrographs; top row); microglia colabeled with FITC (green) conjugated EMB11 (left and middle, bottom panels), and astroglia colabeled with TRITC (red)-conjugated GFAP (right, bottom panel). Bars, 15 μm. (f) Bar graph of mean OD values measured in glial cytoplasm in the nigra of controls (□), IPDs in regions containing intact neurons (■) and IPDs in regions containing fragmenting neurons (▨). iNOS intensity was significantly higher in IPDs in regions containing intact neurons (P = 0.05) as well as in regions containing fragmented neurons (P = 0.5) compared with controls.

which could confer neuroprotection in IPD. We have therefore examined the cellular locations and distribution of Cox-1, Cox-2, LC-1, and iNOS in the human substantia nigra and caudate–putamen of parkinsonian and neurologically healthy subjects postmortem in an effort to understand whether glia are neurodestructive or able to offer protective assistance to failing neurons.

RESULTS

iNOS Immunoreactivity

Substantia nigra. In control nigra iNOS-IR was present in a subpopulation of regularly spaced process-bearing cells (Fig. 1a) which colabeled either with the microglial marker EBM11 (Fig. 1e, middle) or with the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 1e, right). The total number (mean ± SEM) of iNOS-IR astroglia and ramified microglia within 100 μm of intact neurons was 3.54 ± 0.43 (iNOS-IR astroglia and ramified microglia were present in similar numbers). Occasional EBM11-IR amoeboid microglia in the neuropil also contained iNOS-IR (illustrated in Fig. 1e for IPDs; left). However, around 80–90% of the astroglia and ramified microglia populations surrounding intact neurons were not iNOS-IR.

In IPDs, iNOS-IR was also present in ramified and amoeboid microglia and astroglia (Fig. 1b). In nigral

regions containing predominantly intact neurons the total (mean ± SEM) number of process bearing glia within a 100-μm radius of an intact neuron was higher (7.9 ± 1.4) than seen in controls ($0.05 > P > 0.02$) due predominantly to an increase in the number of iNOS-IR ramified microglia with no change in iNOS-IR astroglia. In neuropil-containing neuronal fragments and neuromelanin, numerous clusters of intensely iNOS-IR amoeboid microglia were also present forming temporary glial "scars" (Fig. 1b, indicated by arrowheads). The total number of iNOS-IR glia within a 100-μm radius of a fragmented neuron in IPD was consequently increased further in these scar regions (13.8 ± 0.6; $P < 0.001$). Astroglia did not appear to contribute to these iNOS-IR scars.

The intensity of glial cytoplasmic iNOS-IR was measured in sections taken from IPD and control groups. iNOS-IR intensity was slightly lower in astroglia (mean ± SEM iNOS-IR intensity 105.8 ± 13.6) compared with ramified microglia (144.2 ± 6.64; $0.1 > P > 0.001$) or amoeboid microglia (144.6 ± 8.3; $0.01 > P > 0.001$). Taking all subjects together, an increasing degree of microgliosis, reflected in an increasing number of EBM11-IR microglia, was broadly associated with an increasing number of iNOS-IR glia counted in adjacent sections ($r = 0.918$). Since IPD nigras were characterized by increased numbers of ramified and amoeboid microglia with little change in the number of iNOS-IR astroglia compared with controls, data for iNOS-IR intensity are combined for all glial populations. As illustrated in Fig. 1f, the mean glial cytoplasm iNOS-IR intensity in IPD nigras in regions containing predominantly intact neurons (Fig. 1f, closed bar) and in regions containing neuronal fragments and amoeboid microglial scars (Fig. 1f, hatched bar) was higher than in controls (Fig. 1f, open bar; $P = 0.05$). iNOS-IR was not present in neurons in control or IPD groups (Figs. 1a and 1b).

Caudate–putamen. Pale iNOS-IR was present predominantly in astroglia and occasionally in ramified microglia in the neuropil of the caudate (not shown) and putamen of both control (Fig. 1c) and IPD (Fig. 1d) groups with a similar distribution. Macrophages in blood vessels were also iNOS-IR (data not shown). Amoeboid microglia were seldom present in either group. No significant difference was found in iNOS-IR intensity in process bearing glia between groups in either the caudate (mean ± SEM; controls 43.6 ± 4.2 and IPDs 39 ± 3.31; $0.7 > P > 0.6$) or putamen (controls 46.5 ± 3.8 and IPDs 45.9 ± 6; $P > 0.9$).

Lipocortin-1 Immunoreactivity

Substantia nigra. LC-1-IR was present in intact nigral neurons in controls (Fig. 2a, black precipitate, arrows, easily distinguished from the brown-colored neuromelanin) and IPDs (Fig. 2b, arrows) in a pale neuropil. LC-1-IR intensity, as measured by optical density (OD) values, was similar in intact neurons in controls (mean ± SEM; 89.7 ± 7.3) and IPDs; in the latter this was irrespective of whether neurons were situated in neuropil containing few (85.4 ± 8.9; $0.8 > P > 0.7$) or numerous (103.9 ± 16.2; $0.5 > P > 0.4$) neuron fragments and glial scars (Fig. 2c, arrows). Astrocytes were not LC-1-IR (compare LC-1-IR in Fig. 2c with GFAP-IR in Fig. 2d). Their processes extended over intact neuronal somata in both groups but were withdrawn away from fragmented neuronal somata in IPDs (Figs. 2c and 2d, arrows show neuromelanin fragments) as reported previously (Knott *et al.*, 1999).

Occasionally pale LC-1-IR was present in EBM11-IR amoeboid microglia in neuropil surrounding *healthy intact* neurons in controls (mean ± SEM number within 100 μm of an intact neuron 1.33 ± 0.21) and IPDs (2.1 ± 0.7) with no difference in LC-1-IR intensity between groups (Fig. 2i; $0.4 > P > 0.3$). However, in IPD scars, clusters of strongly LC-1-IR amoeboid microglia were found typically around degenerating ventral tier neurons *within* the astrocytic envelope (Fig. 2c, arrowheads, and Fig. 2d; mean ± SEM number present within 100 μm of fragmenting neurons 8.69 ± 1.95). Infrequently, LC-1-IR ramified microglia were also present in IPDs (Fig. 2j, shows ramified microglia, top; and amoeboid microglia, bottom, for IPDs). LC-1-IR was present around the periphery of amoeboid microglial somata (Figs. 2c and 2e, arrows) and was significantly stronger in IPDs than in controls (Fig. 2i, $0.01 > P > 0.001$). A diffuse LC-1-IR was also present around microglial somata in neuropil containing fragmenting neurons (seen in Fig. 2c) and surrounding blood vessels (Fig. 2e, small arrows) which appeared to be either loosely bound to the cell exterior or secreted into the surrounding neuropil. This was reflected by a higher neuropil LC-1-IR intensity (mean ± SEM; 41.3 ± 9.3) compared with neuropil surrounding intact healthy neurons in IPDs (1.03 ± 4.2; $P < 0.01$) or controls (5.6 ± 4.2; $0.1 > P > 0.001$). In IPDs, EBM11-IR macrophages within blood vessels also contained LC-1-IR (Fig. 2g; mean ± SEM OD 167.9 ± 19.6) and our data do not distinguish between endogenous LC-1-IR amoeboid microglia or macrophages deriving from the vasculature.

LC-1 antibody was not applied to the caudate–putamen because of its limited supply.

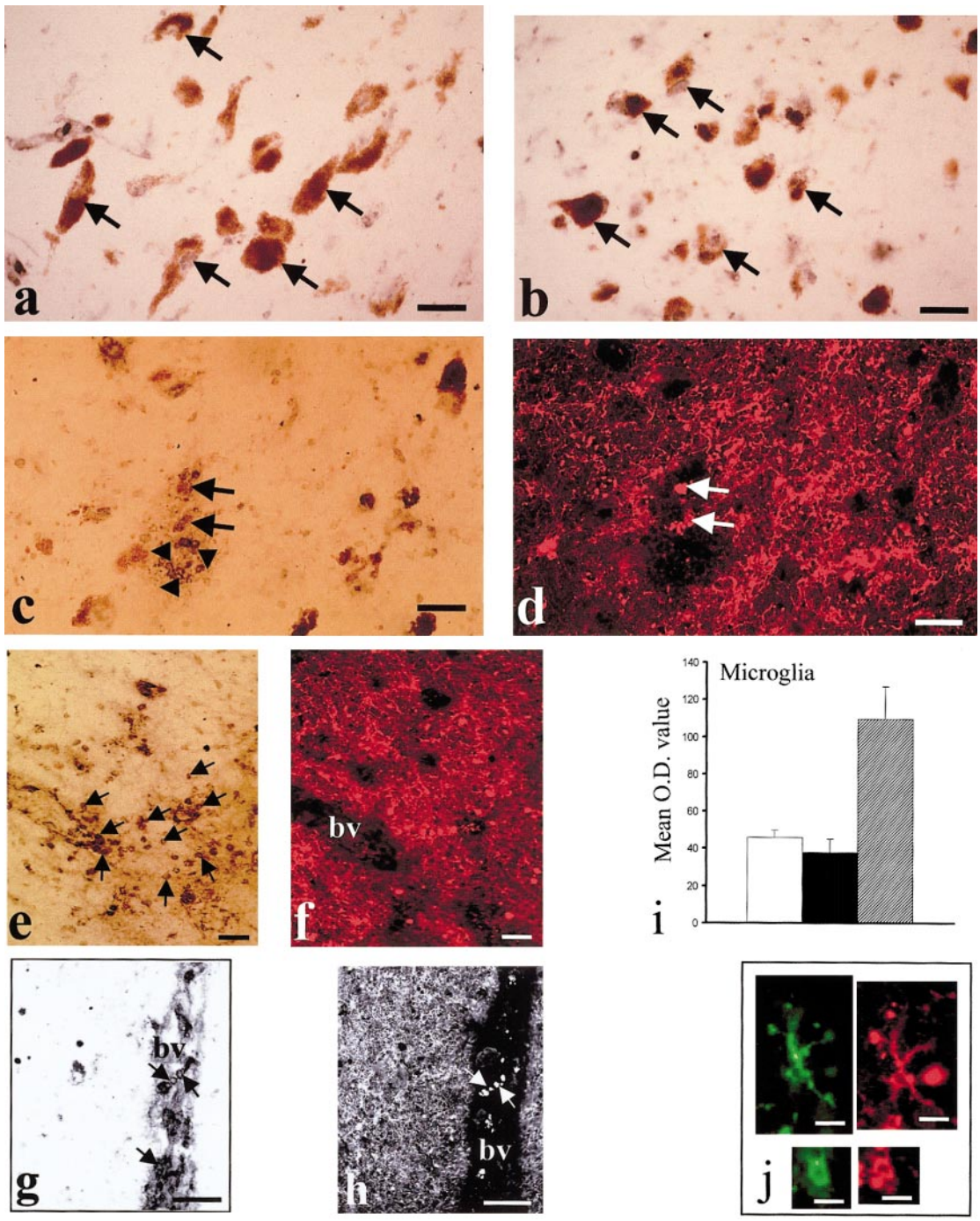


FIG. 2. Light photomicrographs of lipocortin-1 (black precipitate) in control (a) and IPD (b) nigra in regions containing intact neurons (arrowed; containing brown neuromelanin). Paired photomicrographs of lipocortin-1 (bright field; c, e, g) with GFAP (fluorescence, TRITC field; d, f, h). Lipocortin-1-IR microglia (c, arrowheads) are within the astrocytic envelope (d); neuron fragments are arrowed in c and d (neuromelanin fragments are seen in this field because they have endogenous fluorescence). Lipocortin-1-IR is shown in perivascular macrophages / microglia (e, f; arrowed). Lipocortin-1-IR macrophages are also shown within a blood vessel in light (g; arrows) and fluorescence (h; endogenous fluorescence is arrowed) fields. At the left side of the field in g, neuromelanin fragments and a melanized neuron (black) are seen in the neuropil; white fibrils of GFAP are seen in the corresponding fluorescent image. (i) Bar graph of mean OD values measured in microglial cytoplasm in the nigra of controls (□), IPDs in regions containing intact neurons (■) and IPD's in regions containing fragmenting neurons (▨). There was no significant difference in OD values measured between controls and IPDs in regions containing intact neurons ($0.4 > P > 0.3$), but microglia in regions containing fragmented neurons had significantly higher lipocortin-1 intensity compared with controls ($0.01 > P > 0.001$). (j) Ramified (top panels) and amoeboid (bottom panels) microglia colabeled with FITC-conjugated EBM11 (green) and TRITC-conjugated lipocortin-1 (red) taken from IPD sections. Bars in a–h, 45 μm; bars in j, 15 μm.

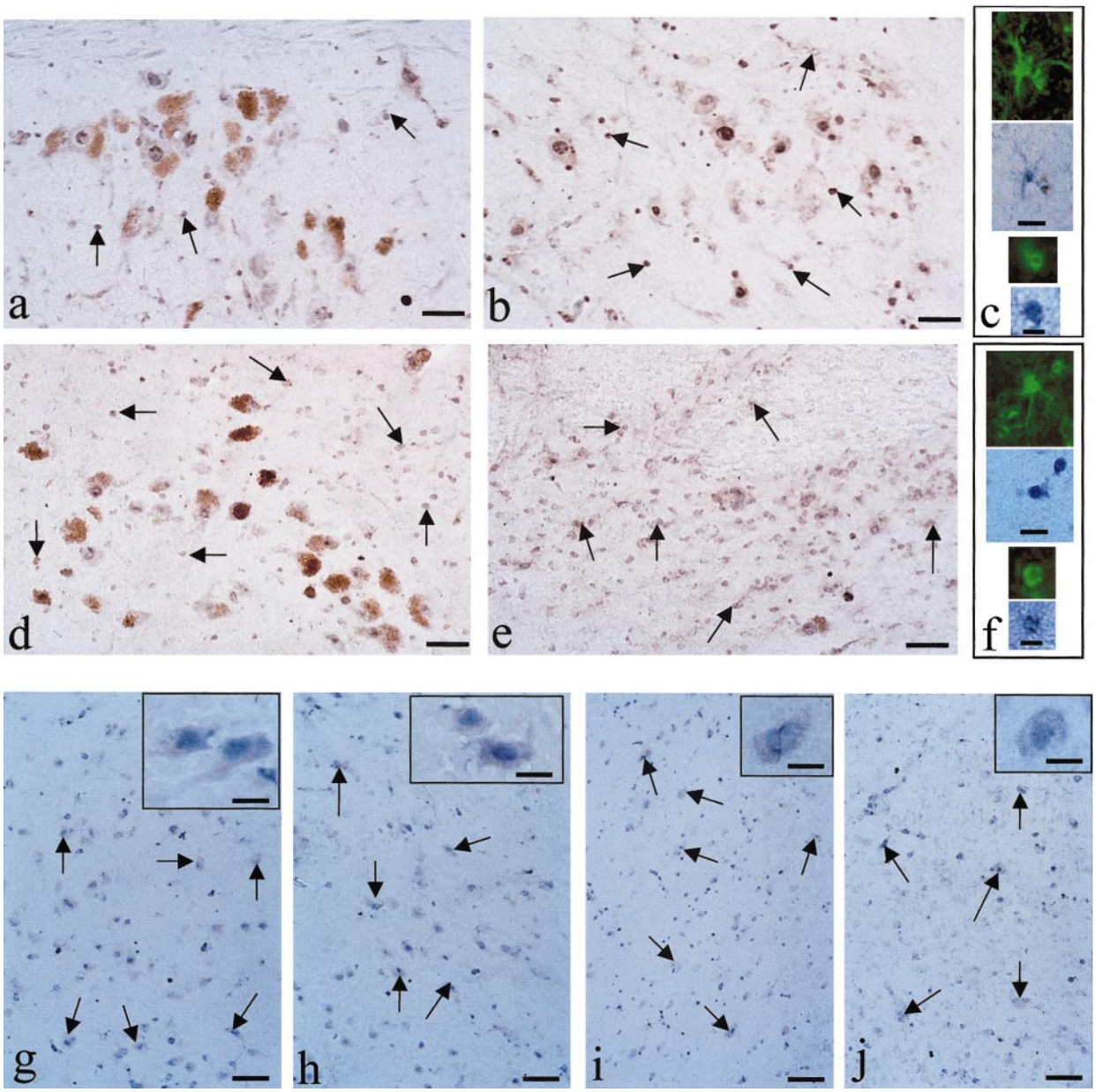


FIG. 3. Photomicrographs of Cox-1-IR in control (a) and IPD (b) nigra (bars, 50 μm) and control (g) and IPD (h) putamen (bars, 150 μm). (c) Light micrographs of representative Cox-1-IR astroglia (top panels) and microglia (bottom panels) colabeled with FITC (green) conjugated to GFAP or EMB11 at higher magnification; bars, 20 μm. Representative photomicrographs of Cox-2-IR in control (d) and IPD nigra (e) (bars, 50 μm) and control (i) and IPD (j) putamen (bars, 150 μm). (f) Light micrographs of Cox-2-IR astroglia (top panels) and microglia (bottom panels) colabeled with FITC (green) conjugated to GFAP or EMB11 taken from IPD sections; bars, 20 μm. Bars of insets in g–j, 50 μm. Glia are arrowed in a, b, d, and e. Neurons are arrowed in g–j.

Cox-1 Immunoreactivity

Substantia nigra. Cox-1-IR was present in melanized and nonmelanized neuronal somata and processes in controls (Fig. 3a) and IPDs (Fig. 3b). In both groups Cox-1-IR intensity in neuronal cytoplasm was moderate (mean OD $\pm$ SEM; for controls 99.1 $\pm$ 12.1, for IPDs 120.8 $\pm$ 7.8; $0.2 > P > 0.1$) and perinuclear stain was present. Astroglia, spaced regularly in the neuropil, contained Cox-1-IR (mean $\pm$ SEM; controls 119.7 $\pm$ 13.4; IPDs 136.2 $\pm$ 4.5, $0.2 > P > 0.1$). In IPDs Cox-1-IR

astroglia (Fig. 3c, top panels) and amoeboid microglia (Fig. 3c, bottom panels; mean ± SEM OD 133.9 ± 2.6) were present in scars containing degenerated neurons resulting in an increased concentration of Cox-1-IR glia in scar regions that was not seen in controls. The neuropil Cox-1-IR intensity was similar in controls (3.6 ± 7.6) and IPDs (10.5 ± 7.8; $0.6 > P > 0.5$). Blood vessel endothelial cells and perivascular EBM11-IR cells that resembled macrophages also contained pale Cox-1-IR (data not shown).

Caudate–Putamen

Numerous somata of small and large neurons and their processes were moderately Cox-1-IR; both cytoplasmic and perinuclear stain was present (illustrated for the putamen; Fig. 3g shows controls and Fig. 3h shows IPDs). There was a similar distribution and intensity of neuronal Cox-1-IR in caudate (mean ± SEM; controls 77.6 ± 6.1; IPDs 80.9 ± 5.8, $0.8 > P > 0.7$) and putamen (controls 77.2 ± 3.5; IPDs 75.3 ± 8.4, $P > 0.9$) for both groups. A similar distribution and intensity of Cox-1-IR astroglia was also found in both groups in the caudate (mean ± SEM; controls 75.7 + 7.8; IPDs 69.5 + 4.7; $0.5 > P > 0.4$) and putamen (controls 71.6 + 4.3; IPDs 79.7 + 6.2; $P = 0.3$). Endothelial cells were Cox-1-IR and process-bearing cells in the neuropil surrounding blood vessels were strongly Cox-1-IR (data not shown).

Cox-2 Immunoreactivity

Substantia nigra. Cox-2-IR was present in melanized and nonmelanized neuronal somata and in their processes in controls (Fig. 3d) and IPDs (Fig. 3e) in a pale neuropil. Neuronal somata cytoplasm contained moderate Cox-2-IR (mean ± SEM; controls 96.0 ± 16.5; IPDs 111.2 ± 17.2; $0.6 > P > 0.5$) and strong perinuclear stain was present in both groups. Astroglial somata cytoplasm, but not processes (Fig. 3f, top panels), also contained Cox-2-IR in both groups (mean ± SEM; controls 115.7 ± 3.6; IPDs 116.5 ± 9.2; $P > 0.9$). In IPD scar regions (Fig. 3e) and around nerve fiber bundles (not shown), amoeboid microglia (Fig. 3f, bottom panels) also contained Cox-2-IR (134 ± 14.0) producing an increase in Cox-2-IR glia in scar regions that was not seen in controls. Astroglia around blood vessels were Cox-2-IR but blood vessel endothelial cells were unstained.

Caudate–Putamen

Numerous small and large neurons contained Cox-2-IR in the caudate (data not shown) and putamen (Figs. 3i and 3j) with no significant difference in intensity in controls (pooled caudate and putamen neurons, mean ± SEM 112.5 ± 6.6) and IPDs (pooled caudate and putamen neurons 106.6 ± 7.1, $0.6 > P > 0.5$). Astroglial somata also contained Cox-2-IR (pooled caudate and putamen data for controls 85 ± 7.9 and for IPDs 81.4 ± 8.2; $0.8 > P > 0.7$). Astroglial end feet around blood vessels contained pale, diffuse Cox-2 IR and macrophages in blood vessels also contained Cox-2-IR (data not shown).

DISCUSSION

The mechanism whereby neuronal loss occurs in idiopathic Parkinson's disease is unknown but excessive production of free radicals such as NO or of proinflammatory prostanoids have been suggested as possible contributory factors (Jenner, 1994; Ebadi *et al.,* 1996; Wilkin and Knott, 1999). We have examined the distribution of potential pro- and anti-inflammatory molecules in the SNc and caudate–putamen in order to assess their contribution to the pathogenesis of Parkinson's disease. Consistent with pathological findings (Braak *et al.,* 1996), there appeared to be little difference in the distribution of iNOS, Cox-1, or Cox-2 in caudate and putamen between groups and the remainder of the discussion focuses on findings in the nigra.

iNOS in IPD

We have characterized the cellular sources of iNOS in control and IPD human nigra. First, we demonstrate that process bearing iNOS-IR cells in controls and IPDs colabeled either for GFAP (i.e., were astrocytes) or for the CD68 epitope EBM11 (i.e., were ramified microglia). The percentage of iNOS-IR astroglia and ramified glia in control and healthy IPD nigra was small (<10–20% of the glial population). Second, we demonstrate that amoeboid microglia surrounding fragmented neurons in IPD nigral scar tissue contain iNOS-IR, as suspected previously (Hunot *et al.,* 1996) and that this is the major iNOS-expressing cell type comprising the scar tissue. Our use of flash-frozen tissue and mild fixation has allowed dual labeling for astrocyte and microglial markers and detection of iNOS in ramified microglia that was not possible by Hunot and colleagues (1996) using the same antibody but harsh fixation conditions.

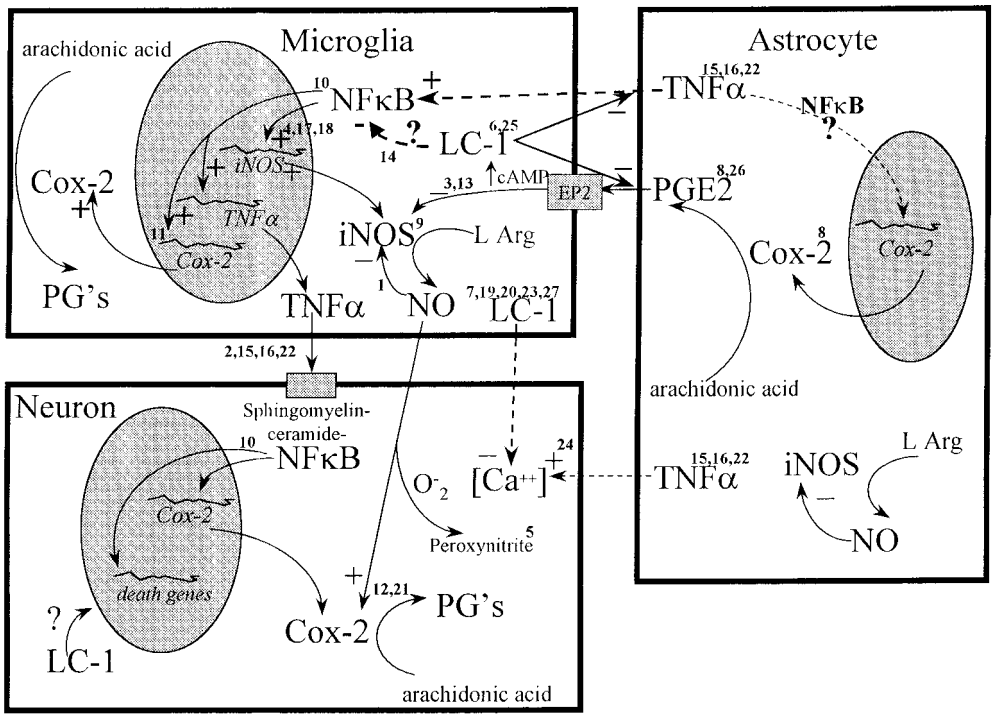
The astrocyte contribution to total glial iNOS was small in our study, in terms of both cellular content and astrocyte number (1–2 per 100 μm^2 tissue area) consistent with findings in culture that microglia produce more NO per cell than astroglia (Boje and Arora, 1992). Our data suggest therefore that amoeboid microglia are the principal source of NO in the IPD nigra. This can have detrimental consequences for compromised neurons (Boje and Arora, 1992; Jenner, 1994; Wilkin and Knott, 1999) since NO reacts with superoxide to form peroxynitrite, a potent oxidant (Dawson *et al.*, 1992). Indeed, NO-dependent neuronal (Chao *et al.*, 1996) or oligodendrocyte (Merrill *et al.*, 1993) injury has been demonstrated in culture. Inhibition of iNOS improved neuronal survival in mixed primary rodent neuron–glial cortical (Dawson *et al.*, 1994) or human whole brain (Chao *et al.*, 1996) cultures. Elevated iNOS expression has also been reported in brain microvessels (Dorheim *et al.*, 1994) and neurons bearing "tangles" (Vodovotz *et al.*, 1996) in Alzheimer's disease and in amoeboid microglia in demyelinating multiple sclerosis lesions (Merrill *et al.*, 1993; Bagasra *et al.*, 1995; De Groot *et al.*, 1997).

It is still unknown whether nigral glia *respond* to neuronal death with the production of various cytokines or whether *alterations in glial function* such as lowered levels of reduced glutathione (Sian *et al.*, 1994) or complex 1 deficiency (Schapira *et al.*, 1990; Beal, 2000), both of which are reported in IPD, *precipitate* the onset or progression of neuronal death. Thus, iNOS could be up-regulated *preceding or following* the death of nigral neurons. Since iNOS-IR microglia congregate predominantly in scar areas where neurons have already died or are likely to be highly compromised, it is possible that iNOS is expressed in response to signals from vulnerable or failing neurons such as extracellular potassium, ATP, or cytokines. Subsequent glial responses, illustrated in Fig. 4, which shows our speculations on potential glial–neuronal interactions in IPD nigral scars based on our findings and current literature consensus, might include TNF-α release (Sawada *et al.*, 1989). Elevated expression of TNF-α has been reported in Parkinson's disease (Mogi *et al.*, 1994a,b) and TNF-α receptors are present on nigral dopaminergic neurons in IPD (see Fig. 4) but not healthy neurons (Boka *et al.*, 1994). TNF-α can activate the sphingomyelin-dependent transduction pathway in dopaminergic neurons resulting in nuclear translocation of the transcription factor, nuclear factor κB (NFκB), of which there is evidence in IPD (Hunot *et al.*, 1997). In rodent mesencephalon cultures this was followed by production of oxygen free radicals and neuronal death (Hirsch *et al.*, 1999). TNF-α also induces iNOS in glia (Park *et al.*, 1994; Chartrain *et al.*, 1994) which could compound neuronal damage mediated by NO. A further consequence of elevated NO may be activation of the Cox catalytic site (Salvemini *et al.*, 1993; Landino *et al.*, 1996) which could increase prostanoid production particularly in CNS regions with reduced vascular perfusion. The primary function of iNOS-IR glia in IPD therefore might be to promote neuronal disintegration and removal of debris but this could contribute to disease progression and propagation to adjacent healthy areas. The destructive cascade is probably self-limiting, however, since NO is a negative feedback inhibitor of iNOS transcription (Park *et al.*, 1994, 1997; Murphy, 2000) and enzyme activity (Assreuy *et al.*, 1993).

Cyclooxygenases in IPD

We have demonstrated the presence of Cox-1-IR and Cox-2-IR in neuronal and glial populations in neurologically healthy and IPD nigra. Cox-1 is thought to be constitutively expressed (O'Neill and Hutchinson, 1993) but Cox-2 expression has been demonstrated in neurons following ischemia (Nogawa *et al.*, 1997) and up-regulated during seizures (Marcheselli and Bazan, 1996) and is thought in these circumstances to be involved in excitotoxicity. Interestingly, in Alzheimer's disease, cortical neurons (Yasojima *et al.*, 1999) and astroglia were found to contain Cox-2 and administration of nonsteroidal anti-inflammatory drugs reduced the risk of dementia (Pasinetti and Aisen, 1998). In our study, there was no difference in neuronal Cox-1 or Cox-2 immunoreactivity between controls and IPDs. Therefore, although the NFκB consensus sequence is present in the human Cox-2 gene (see Fig. 4; Kosaka *et al.*, 1994) it is unlikely that NFκB-mediated up-regulation of Cox-2 mRNA occurs in dopaminergic neurons in IPD. Our data do not preclude the possibility of alterations in cyclooxygenase *activity* (e.g., see Fig. 4) or of downstream changes in prostanoid synthesis in dopaminergic neurons, however.

In agreement with our findings, Cox-2 was demonstrated in normal and reactive astrocytes following cerebellar kainate lesion in rodents (Hirst *et al.*, 1999) and in perivascular macrophages surrounding hippocampal bacillus Calmette–Guerin lesion (Minghetti *et al.*, 1999a), with little change in neuronal Cox-2 in both studies. One potential product of glial Cox-2 is PGE_2, the levels of which double in line with Cox-2 mRNA levels following cerebellar kainate lesion in rodents (Hirst *et al.*, 1999). PGE_2 release may be stimulated by agents such as bradykinin or ATP in astro-



Speculative glial-neuronal interactions in scar areas of IPD nigra.

FIG. 4. Schematic diagram of potential neuronal–glial biochemical interactions that might occur in the IPD nigra, based on our findings and those of others. Following neuronal damage astroglia might respond by releasing TNF-α which can activate neuronal and possibly microglial TNF-α receptors. In IPD scars, microglia might respond by up-regulating TNF-α, iNOS, and Cox-2 transcription, leading to an elevated synthesis of prostanoids and NO and TNF-α release which could promote neuronal demise and disease progression. Neurons might respond to glial TNFα by up-regulating "death genes" and possibly Cox-2 synthesis (although the latter is unlikely). Lipocortin-1 (LC-1) is synthesized by amoeboid microglia local to nigral neurons in IPD and this anti-inflammatory molecule has a plethora of effects including reduction in the release of glial TNF-α and of PGE_2, inhibition of iNOS, and buffering of neuronal intracellular calcium. It might therefore put a brake on a local inflammatory reaction in IPD. References pertinent to this figure are indicated numerically as follows: 1, Assreuy *et al.*, 1993; 2, Boka *et al.*, 1994; 3, Caggiano and Kraig, 1999; 4, Chartrain *et al.*, 1994; 5, Dawson *et al.*, 1992; 6, De Caterina *et al.*, 1993; 7, Flower, 1998; 8, Hirst *et al.*, 1999; 9, Hunot *et al.*, 1996; 10, Hunot *et al.*, 1997; 11, Kosaka *et al.*, 1994; 12, Landino *et al.*, 1996; 13, Minghetti *et al.*, 1997; 14, Minghetti *et al.*, 1999b; 15, Mogi *et al.*, 1994a; 16, Mogi *et al.*, 1994b; 17, Murphy and Grzybicki, 1996; 18, Park *et al.*, 1994; 19, Philip *et al.*, 1997; 20, Raynal and Pollard, 1994; 21, Salvemini *et al.*, 1993; 22, Sawada *et al.*, 1989; 23, Solito *et al.*, 1993; 24, Soliven and Albert, 1992; 25, Sudlow *et al.*, 1996; 26, Wilkin and Marriott, 1993; 27, Young *et al.*, 1999.

glial cultures (Hirst *et al.*, 1999) and exogenous PGE_2 activation of EP_2 receptors down-regulates iNOS in activated microglial cultures (Minghetti *et al.*, 1997). In this manner, astroglia ensheathing neurons adjacent to activated microglia in IPD nigra might protect *healthy* neurons from microglial attack (see Fig 4). Glial scars, present only in IPD nigras, contained amoeboid microglia that expressed both Cox isoforms. Thus, a greater potential for prostanoid synthesis is attributed to amoeboid microglia here. The nature of these prostanoids is unknown as is their action, which may be either neuroprotective or neurodestructive to vulnerable neurons.

LC-1 in IPD

To our knowledge this is the first report demonstrating LC-1-IR in the human nigra. LC-1-IR was present in similar amounts in neurons in normal healthy and IPD nigra. Its synthesis does not therefore appear to be up-regulated in failing or stressed neurons but a perinuclear localization suggests that it might modify the level of gene transcription for intracellular proteins with consequent beneficial downstream effects. An important source of LC-1 in the IPD nigra might be the amoeboid microglia surrounding failing neurons. LC-1 may be released from these microglia into the neuropil

covering vulnerable neurons, as seen in the KA-lesioned rodent cerebellum and in microglial cultures (Young *et al.*, 1999), where it might exert neuroprotective effects at the neuronal cell membrane. Alternatively, it might act in an autocrine manner to dampen down NO and prostanoid production (see Fig. 4) as found in lipopolysaccharide-stimulated rodent microglial cultures (De Caterina *et al.*, 1993; Minghetti *et al.*, 1999a,b,c), perhaps by inhibiting NFκB activity. This would limit the extent of NO and prostanoid-mediated damage. In addition, LC-1 is thought to inhibit phospholipase A_2 activity (Flower, 1988) and thereby prevent arachidonic acid release which is a prerequisite for prostanoid synthesis.

Lipocortin-1 protects against ischemic (Relton *et al.*, 1991) and NMDA-mediated CNS damage (Black *et al.*, 1992) and exerts antipyretic activity (Carey *et al.*, 1990; Strijbos *et al.*, 1992; Davidson *et al.*, 1991) *in vivo* in the rodent, situations in which the proinflammatory ILs IL-1β, IL-8, and IL-6 are implicated (Strijbos *et al.*, 1992; Rothwell and Relton, 1993). Interleukin-1β and IL-6 are elevated in the caudate–putamen and cerebrospinal fluid in IPD (Mogi *et al.*, 1994a; Müller *et al.*, 1998), CSF levels of IL-6 correlating inversely with symptom severity in drug-naive patients (Müller *et al.*, 1998). IL-1β is also a recognized inducer of iNOS and Cox-2 (LaPointe and Isenovic, 1999) and it is therefore conceivable that LC-1 has the potential to exert anti-inflammatory actions by inhibiting certain effects of interleukins in IPD.

LC-1 has been demonstrated in macrophages and astrocytes in autoimmune encephalomyelitis lesion in Lewis rats (Huitinga *et al.*, 1998) and in human multiple sclerosis CNS lesions (Elderfield *et al.*, 1992). An upregulation of LC-1 was also detected in blood vessels of KA-lesioned rodent cerebellum (Young *et al.*, 1999). The function of LC-1 in perivascular and vascular macrophages in these situations and in human IPD nigra might be to impede neutrophil and monocyte recruitment as suggested previously (Young *et al.*, 1999) which would temper an inflammatory onslaught. In addition, LC-1 suppressed the release of both TNF-α and PGE_2 from stimulated human peripheral mononuclear cells (Sudlow *et al.*, 1996). If this occurs in the IPD nigra (as speculated in Fig. 4). LC-1 could potentially provide an effective means of limiting an inflammatory cascade to the immediate vicinity of dying neurons.

CONCLUSIONS

The sequence of events that accompanies the death of a vulnerable neuron in IPD is unknown. However, it is likely that local astroglia and ramified microglia respond to as yet uncharacterized neuronal signals by synthesizing neurotrophins such as GDNF, NT-3, or BDNF in an attempt to assist a failing neuron (Abiru *et al.*, 1998; Heese *et al.*, 1988; Beck *et al.*, 1995; Langan *et al.*, 1995; Batchelor *et al.*, 1999). If these measures are unsuccessful, astroglial processes withdraw from the neuronal somata (and axons). This has been shown to induce apoptosis of cultured cortical neurons (Ohgoh *et al.*, 1998) perhaps due to increased oxidative stress resulting from the removal of glutathione peroxidase (Damier *et al.*, 1993). Signals which recruit microglia to failing neurons are unknown; however, astroglial-derived neurotrophins may be involved in microglial activation as shown in culture (Elkabes *et al.*, 1996). LC-1-IR microglia are found within the astroglial envelope in close association with degenerating neurons in IPD. Secretion of this anti-inflammatory and neuroprotective molecule might provide a final attempt at a neuronal rescue. If the neuron continues to deteriorate and upregulates TNF-α receptors, neuronal demise is probably precipitated by the effects of destructive cytokines such as TNF-α, NO, certain prostaglandins, and leukotrienes released from local glia, particularly amoeboid microglia, which now take on a phagocytic role to remove neuronal debris from IPD nigral scars.

EXPERIMENTAL METHODS

Clinical Details

Substantia nigra from 11 patients with a postmortem neuropathological diagnosis of idopathic Parkinson's disease (IPD group, aged 67 to 84 years; 7 male) and 10 subjects with no neurological disease (control group, aged 63 to 85 years; 5 male) postmortem were studied. Clinical details and causes of death are listed in Table 1. The postmortem delay interval for both groups ranged from 4.83 to 47.5 h. There was no significant difference in age ($P > 0.9$) or postmortem delay between groups ($P > 0.9$). All subjects underwent a full postmortem neuropathological examination to confirm the clinical diagnosis. All IPD cases showed marked cell loss in the SN and locus coeruleus with Lewy bodies identified in the brain stem, subcortical nuclei, and cerebral cortex. Alternative causes of parkinsonism as well as other neurological diseases were excluded. Two cases (5 and 6) had a diagnosis of IPD with Alzheimer's like dementia. All IPD patients received and responded to levodopa (190–750 mg/day); individual doses and other drugs are detailed in Table 1.

TABLE 1
Table of Clinical Data from Patients and Controls in the Nigral Study

Case	Sex	Age (years)	PM delay (h)	Diagnosis	Duration (years)	Cause of death	Drugs (mg/24 h)		
							l-DOPA	Selegiline	Others
1	F	82	24	IPD	7	Nat. causes	190	10	Bromocriptine, 2.5; melleril, 10
2*	F	80	11	IPD	16	Nat. causes	400 Madopar	5	Milpar, 40; sulpiride, 100; pergolide, 3
3*	M	67	33.5	IPD	17	Unknown	950 Sinemet	10	Fluoxetine, 10
4	M	74	4.83	IPD	6	Bronchopneumonia	500 Sinemet Plus		
5	M	74	47.5	IPD + AD	3	Bronchopneumonia	750		
6*	F	75	28.15	IPD + AD	12	Hemopericardium	500		
7	M	71	5	IPD	21	?CVA	1800 co-careldopa		$FeSO_4$
8	M	73	24	IPD	9	Bronchopneumonia	400		
9	M	75	17	IPD	20	Pyrexia, Nat. causes	500 Madopar CR	5	
10	M	84	40	IPD	7	Chest infection	Unknown		
11	F	82	49.5	IPD	19	IHD	250		
12	M	67	22	CON		MI			
13*	F	85	34	CON		Breast Ca.			
14	M	77	27.5	CON		Pancreatic Ca.			
15*	F	73	28	CON		Bronchial Ca.			
16*	F	81	19.5	CON		Rupt. thor. aneurism			
17	F	77	18	CON		Unknown			
18	M	75	34	CON		MI			
19	F	84	18	CON		LVF			
20	M	63	26	CON		MI			

Note. IPD, idiopathic Parkinson's disease; AD, Alzheimer's-like dementia; CON, control; Nat. causes, natural causes; CVA, cerebrovascular accident; IHD, ischemic heart disease; MI, myocardial infarction; Ca., carcinoma; Rupt. thor. aneurysm, ruptured thoracic aneurysm; LVF, left ventricular failure.

Caudate–putamen from 10 subjects with IPD (aged 67 to 88 years, 6 male) and 8 control subjects (aged 43 to 86 years, 3 males) were also studied. There was no significant difference in age ($0.5 > P > 0.4$) or postmortem delay between groups ($0.5 > P > 0.4$). Clinical and pathological data is summarized in Table 2. Six subjects (asterisked in the tables) are common to both studies. No information on drug intake was available for control subjects.

Tissue Preparation

Brains were collected and divided midsagittally. One half was fixed in paraformaldehyde for neuropathological examination and the other was dissected into discrete brain regions, flash frozen. and stored at −70°C. Sections (12 μm thick) were cut at −20° from the substantia nigra or caudate–putamen and stored at −70°C until processed for immunohistochemistry.

Immunohistochemistry

Tissue sections were thawed at room temperature (around 21°C) and fixed in methanol at −20°C for 2 min (lipocortin-1, Cox-1, and Cox-2) or 5 min (iNOS) which produced the greatest intensity and cellular distribution of stain. After phosphate-buffered saline (PBS) wash, endogenous peroxidase activity was quenched with hydrogen peroxide (3%) for 30 min and nonspecific staining was blocked in swine serum (5%) for a further 30 minutes at 21°C. Sections were incubated at 4°C for up to 48 h in (a) rabbit anti-lipocortin-1 polyclonal antibody raised against amino acids 15–31 within the N terminus of human LC-1 (at 1/500 in PBS; Becherucci *et al.*, 1993), (b) anti-iNOS IgG2a mouse monoclonal antibody (at 1/25 in PBS; Transduction Labs), (c) rabbit anti-ovine Cox-1 polyclonal antibody raised against a synthetic peptide from ovine prostaglandin G/H synthase-1 (PGHS-1), which did not recognize PGHS-2 (Cayman Chemicals; PGHS-1; 160108; 1/250 in PBS) but produced the same pattern of staining as goat anti-human Cox-1 polyclonal antibody (Santa Cruz; sc-1752; data not shown), or (d) rabbit anti-Cox-2 polyclonal antibody raised against amino acids 584–598 within the C terminal of murine/human PGHS-2 (1/100 in PBS; DeWitt and Meade, 1993).

Polyclonal antibodies were visualized with goat anti-

TABLE 2
Table of Clinical Data from Patients and Controls in the Caudate–Putamen Study

Case	Sex	Age (years)	PM delay (h)	Diagnosis	Duration (years)	Cause of death	Drugs (mg/24 h)		
							l-DOPA	Selegiline	Others
1	M	71	38	IPD	15	Bronchopneumonia	400		
2*	F	80	11	IPD	16	Nat. causes	400 Madopar	5	Milpar, 40; sulpiride, 100; pergolide, 3
3*	M	67	33.5	IPD	17	Unknown	950 Sinemet	10	Fluoxetine, 10
4	M	81	11.5	IPD	5	RF, Prostate Ca.	500	5	
5	M	82	9	IPD	6	Bronchopneumonia	500		
6*	F	75	28.15	IPD + AD	12	Hemopericardium	500		
7	M	76	26.5	IPD	19	IHD, MI	650	10	Benzhexol, 2
8	M	76	20.5	IPD	8	COAD	880		
9	F	88	19.75	IPD	17	Pneumonia	750	10	
10	F	73	20	IPD	15	Cardiac event	350		
11	F	83	22	CON		Lung Ca.			
12	F	53	29.5	CON		IC hemorrhage			
13*	F	85	34	CON		Breast Ca.			
14	M	86	53	CON		Bronchopneumonia, HF			
15*	F	73	28	CON		Bronchial Ca.			
16*	F	81	19.5	CON		Rupt. thor. aneurism			
17	M	74	5.5	CON		LVF			
18	M	43	15	CON		Heart attack			

Note. IPD, idiopathic Parkinson's disease; AD, Alzheimer's-like dementia; CON, control; Nat. causes, natural causes; RF, renal failure; Ca., carcinoma; IHD, ischemic heart disease; MI, myocardial infarction; LVF, left ventricular failure; COAD, chronic obstructive airways disease; IC, intracerebral (hemorrhage); HF, heart failure.

rabbit IgG conjugated to horseradish peroxidase (1 μg/ml in PBS; Vector Labs) and the monoclonal antibody with horse anti-mouse IgG conjugated to horseradish peroxidase (3.5 μg/ml in PBS; Vector Labs) for 60 min at 21°C followed by 3′,3-diaminobenzidine (0.05%) with nickel enhancement according to the manufacturer's instructions (Vector Labs). Sections were then incubated further in either anti-GFAP monoclonal antibody (1/400 in PBS; Sigma) or anti-GFAP polyclonal antibody (1/500 in PBS; DAKO) as appropriate, overnight at 4°C, and visualized with either Texas red-conjugated horse anti-mouse antibody (4 μg/ml in PBS; Vector Labs) or rhodamine-conjugated swine anti-rabbit secondary antibody (1/300 in PBS; DAKO), respectively, for 60 min at 21°C. Certain sections were dual labeled with EBM11 IgG1κ which recognizes the CD68 epitope (1/100 in PBS; DAKO) rather than anti-GFAP antibodies and visualized with fluorescein conjugated to either rabbit anti-mouse IgG1 (1/100 in PBS; Serotec) or to horse anti-mouse IgG (1/250 in PBS; Vector Labs) as secondary antibody. Sections were mounted in Cityfluor for fluorescence and light microscope examination using a Reichert Jung Polyvar microscope. Photomicrographs of sections were taken with Fujichrome 64 tungsten and 400 daylight films.

Antibody Controls

Antibody specificity was demonstrated by Western blot against purified LC-1 (5 μg; kind gift from Dr. E. Solito), Cox-1, Cox-2, and iNOS (0.5 μg; Cayman Chemicals) proteins and the data, shown in Fig. 5, are in accord with previously published findings (Breder *et al.*, 1995; Young *et al.*, 1999) or manufacturer's data sheets.

Immunohistochemical controls were performed by omission of primary antibodies and in no case was any stain seen in the absence of primary antibody. Further controls were performed by overnight incubation of the antisera with 100 times molar excess of the appropriate purified peptide at 4°C, prior to staining tissue sections. The characteristic pattern of staining was completely abrogated under these conditions (data not shown). In addition staining was demonstrated in tissues known to contain mRNA for the respective antigen but not in tissues known not to contain the mRNA (for relevant mRNA and protein information see Deininger *et al.*, 1999; Flower and Rothwell, 1994; Hirst *et al.*, 1999; Strijbos *et al.*, 1990; Yasojima *et al.*, 1999; Young *et al.*, 1999). For example, iNOS-IR could be demonstrated in a human glioma specimen but did not stain human or rodent CNS neurons or peripheral tissues such as skel-

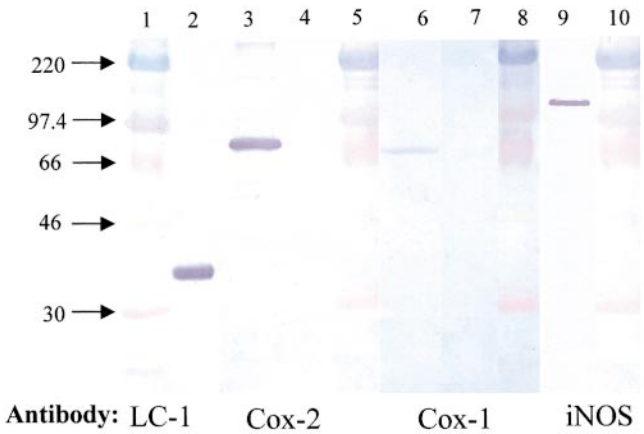


FIG. 5. Western blot of purified proteins for LC-1 (5 μg; lane 2, 37 kDa), Cox-2 (0.5 μg; lanes 3 and 7, 72 kDa), Cox-1 (0.5 μg; lanes 4 and 6, 70 kDa), and iNOS (10 μg; lane 9, 130 kDa). Molecular weight markers are in lanes 1, 5, 8, and 10 and weights are indicated on the right of the figure. Proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% SDS–PAGE), blotted onto nitrocellulose, and incubated with antibodies (indicated at the bottom of the figure) at the concentrations used for staining tissue sections. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (as used for sections) and chloronaphthol. MW, molecular weight marker, kDa.

etal muscle cells (data not shown). Cox-1, which stained human cortical and nigral neurons and rodent CNS neurons and kidney, stained human glioma very poorly and did not stain rodent skeletal muscle cells (data not shown). Cox-2, which stained human CNS neurons, human glioma, rodent kidney (data not shown), and kainate-lesioned cerebellum (Hirst *et al.*, 1999), did not stain rodent skeletal muscle cells. Lipocortin-1 stained human glioma poorly (data not shown) and rodent CNS (Mullens *et al.*, 1994; Young et *al*,. 1999), but did not stain rodent skeletal muscle cells (data not shown).

Data Analysis

In order to make objective semiquantitative comparisons of the relative stain intensity of neuronal somata, glial somata and neuropil in control and IPD groups sections were photographed under the same lighting and magnification conditions. The transparencies were scanned with no alteration in image by a Nikon Coolscan II. The digitized images were saved in TIFF format and analyzed by Scion Image (NIH public domain software) program. Five OD measurements of each region of interest were averaged per image. Background readings of unstained regions in each image were subtracted from all other values. In addition, identical photographs were taken on different photographic films to assess for any effects of film processing on OD. There was no significant difference in background OD values (mean $\pm$ SEM OD units) for IPDs (22.5 $\pm$ 1.04) or controls (20.5 $\pm$ 0.22; all values combined 21.6 $\pm$ 0.7) and no effect of film processing on OD measurements (data not shown). Data, expressed as means $\pm$ SEM, are presented for OD values measured in regions of interest for iNOS, LC-1, Cox-1, and Cox-2-like IR in the substantia nigra and caudate–putamen. Statistical analyses were performed using Student's *t* test.

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