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Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease

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Summary. Depletion of reduced glutathione occurs in the substantia nigra in Parkinson's disease and in incidental Lewy body disease (presymptomatic Parkinson's disease) which may implicate oxidative stress in the neurodegenerative process. In this study mercury orange fluorescent staining and immunostaining with an antibody to reduced glutathione have been used to determine the distribution of reduced glutathione in the substantia nigra in Parkinson's disease compared with normal individuals.

Mercury orange staining showed moderate background levels of fluorescence in the neuropil in both control and Parkinson's disease substantia nigra and localised reduced glutathione to the somata of melanized nigral neurons and glial elements of the neuropil. Neuronal nuclei revealed a relative lack of fluorescence after mercury orange staining. There was a significant depletion of reduced glutathione in surviving neurons in Parkinson's disease compared to nerve cell populations in control tissue. Mercury orange fluorescence indicated a high concentration of reduced glutathione in a subpopulation of non-neuronal cells, most likely astrocytes or microglia.

Immunohistochemical examination of nigral tissue from the same Parkinson's disease and control patients with an antibody to glutathione showed staining in neuronal perikarya and axonodendritic processes of melanized nigral neurons which was generally most intense in control neurons. Moderately intense staining of the background neuropil, most prominent in control nigras, and staining of capillary walls was also detected. Intense staining was seen in cells with the morphological features of glial cells in both control and PD nigra.

These data show a significant presence of reduced glutathione in the cell bodies and axons of nigral neurons. They are in agreement with biochemical studies showing depletion of reduced glutathione in substantia nigra in

Parkinson's disease, and indicate a significant loss of neuronal reduced glutathione in surviving nigral neurons in Parkinson's disease.

Keywords: Parkinson's disease, substantia nigra, glutathione, oxidative stress.

Abbreviations

GFAP glial fibrillary acidic protein, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *ILBD* incidental Lewy body disease, *PBS* phosphate-buffered saline, *PD* Parkinson's disease.

Introduction

The cause of the progressive nigral cell degeneration in Parkinson's disease (PD) remains unknown. However, post mortem studies of the substantia nigra strongly suggest the involvement of oxidative stress (Jenner et al., 1992a,b). Oxidative stress may arise from the metabolism of dopamine with the production of potentially harmful free radical species (Dexter et al., 1991; Jenner et al., 1992a,b). This may be important as surviving neurons increase dopamine turnover to compensate for diminishing synaptic transmission. Endogenous or environmental toxins possibly induce oxidative stress through impairment of mitochondrial function and/or free radical formation (Jenner et al., 1992b).

Neuronal degeneration in PD may result from an increased exposure to free radicals coupled with a deficit of antioxidant mechanisms. Most antioxidant systems in the substantia nigra in PD are either unchanged (glutathione peroxidase, catalase, ascorbic acid, α-tocopherol) or increased (superoxide dismutase) in activity (Marttila et al., 1988; Saggu et al., 1989; Sian et al., 1994b). However, there is a marked depletion of reduced glutathione (GSH) and total glutathione in the substantia nigra without any corresponding increase in the oxidant (GSSG) form (Perry et al., 1982, 1986; Riederer et al., 1989; Sian et al., 1994a). The reduction of GSH is selective for the nigra in PD, and does not occur in other neurodegenerative disorders exhibiting similar degrees of nigral cell loss, such as multiple system atrophy and progressive supranuclear palsy (Sian et al., 1994a). The nigral GSH deficit in PD points to oxidative stress as being particularly relevant to neuronal death. GSH loss in PD is also accompanied by a reduction in mitochondrial complex I (NADH CoQ reductase) activity, which is similarly regionally selective for the nigra in PD and does not occur in related basal ganglia degenerative disorders (Schapira et al., 1990).

The importance of GSH depletion to the pathogenesis of PD is further highlighted by a similar degree of nigral GSH depletion found in the related condition of incidental Lewy body disease (ILBD) (Dexter et al., 1994). ILBD occurs in 10–15% of brains from neurologically normal individuals over the age of 60 years and is a pathological diagnosis based on nigral neuronal loss and the presence of Lewy body inclusions (Forno, 1969; Gibb, 1986; Gibb and Lees, 1988). ILBD may represent a preclinical form of PD with a similar nigral pathology, distinctive from that of normal aging (Forno, 1969; Gibb, 1986). The presence of decreased nigral GSH levels at this early stage of PD pathol-

ogy without any alteration in iron levels or complex I activity suggests that an abnormality of glutathione formation or metabolism may be an early component of the disease process.

There is no evidence for a profound alteration in glutathione-related enzymatic processes in PD to explain the nigral GSH depletion. Nigral glutathione peroxidase activity is unaltered (Sian et al., 1994b) implying no change in redox cycling, though midbrain glutathione peroxidase, important in scavenging hydrogen peroxide, is sparsely present in SN pars compacta relative to other local midbrain regions and in PD increased numbers of cells resembling astrocytes immunoreactive for glutathione peroxidase surround surviving nigral neurons (Damier et al., 1993). Normal activity of the glutathione synthetic enzyme, γ-glutamylcysteine synthetase in the nigra in PD suggests that the decrease of GSH content is unlikely to be due to a failure of synthetic mechanisms (Damier et al., 1993). Nigral glutathione transferase activity is also unchanged so utilization via conjugative reactions producing mercapturic acid is unlikely (Perry and Yong, 1986; Sian et al., 1994b). However, there is a selective elevation of nigral γ-glutamyltranspeptidase activity in PD but not in multiple system atrophy, which may be related to GSH depletion (Sian et al., 1994b).

It seems unlikely, therefore, that a primary defect in GSH synthesis or enzymatic handling initiates the onset of the disease process, but excessive consumption and depletion of GSH in the face chronic oxidative stress may represent a susceptibility factor which in some individuals leads to cell death and the occurrence of clinical PD or ILBD.

Identification of the specific regional and cellular distribution of changes in GSH concentration in the nigra in PD may therefore provide insight into the nature of the pathogenesis and progression of PD. Histochemical localisation of GSH in rodent and subhuman primate brain using mercury orange and o-phthaldialdehyde histofluorescence has suggested that it is predominantly present in the neuropil, glial cells, neuronal axonodendritic processes and terminals; most neuronal somata show little if any staining, although the nigra was not specifically examined in these reports (Philbert et al., 1991; Slivka et al., 1987). If nigral neurons contain little GSH, this may render them more vulnerable to oxidative stress.

In the present study, we have employed mercury orange histofluorescence (Asghar et al., 1975) and immunostaining with an antibody specific for glutathione (Hjelle et al., 1994) to determine the distribution of GSH in the substantia nigra of PD cases and of age-matched normal controls. Mercury orange staining is selective for GSH under the conditions used for this study, since mercury orange rapidly reacts with the thiol groups of GSH (<5 minutes) compared to thiol groups of proteins (8 hours) (Asghar et al., 1975). The antibody to glutathione reacts with reduced glutathione (GSH) and has some affinity for the oxidized form (GSSG) (Hjelle et al., 1994), but since GSH is far more abundant than GSSG and levels of GSSG are not increased in the PD nigra (Sian et al., 1994a), alterations in the pattern of immunostaining should primarily relate to alterations in nigral GSH content.

Experimental procedures

Brain tissue samples

Human nigral specimens from age-matched normal controls (n = 6; mean age = 74.8) and cases (n = 6; mean age = 77.0) with histologically proven PD from the UK Parkinson's Disease Society Brain Bank were used in the study. The diagnosis of PD was based on finding a clear depletion of nigral pigmented neurons with Lewy body inclusions in some of the remaining nerve cells and a normal appearance in the striatum. Details of the age-matched control and PD cases are summarised in Table 1. The mean duration of disease in the PD group was 15 years and all patients had received L-DOPA-containing preparations. The mean post mortem interval (i.e. time between death and removal of brain) was 21.3 hours for control brains and 17.2 hours for PD cases. Sections (12 μm) of flash-frozen substantia nigra from normal controls and PD cases were mounted on glass slides. The sections were kept at −70°C until they were stained either with mercury orange or used for immunohistochemical study.

Mercury orange histofluorescence

The mercury orange staining procedure was adapted from Ashgar et al. (1975). Nigral sections mounted on glass slides were immersed for 5 minutes in ice cold (4°C) 50 mM mercury orange dissolved in toluene. The slides were cleared in cold toluene (4°C) and mounted with Permount. Subsequently all stained slides were stored at 4°C and examined within 24 hours of staining.

Phase contrast and fluorescent images were viewed under an Olympus microscope equipped for epifluorescence miscroscopy and photography. Fluorescing images were obtained using an Olympus excitor filter ($450 < \lambda < 490$, emission filter barrier $\lambda = 600$ nm). Photographs were taken with Kodak Tri-X Pan film using 1 minute exposures.

The intensity of fluorescence in melanized nigral neurons relative to background fluorescence of the neuropil was assessed in semi-quantitative fashion. Individual neurons seen in control and PD nigra were graded blind to diagnosis on a scale from 3+ to 3− with respect to the intensity of fluorescence against the background neuropil at 200× magnification.

Table 1. Patient data for control and Parkinson's disease cases

Case	Age	Sex	P.M.I.	C.O.D.
C1	77	F	18	unknown
C2	75	M	5.5	left ventricular failure
C3	73	F	15	congestive heart failure
C4	82	M	33	pulmonary embolism
C5	67	M	22	myocardial infarction
C6	75	M	34	myocardial infarction
P1	82	F	23.5	myocardial infarction
P2	67	M	20	bronchopneumonia
P3	72	F	16	bronchopneumonia
P4	74	M	4.8	bronchopneumonia
P5	86	F	24	unknown
P6	81	F	15	bronchopneumonia

C control, *P* Parkinson's disease, *P.M.I.* post mortem interval (hours), *C.O.D.* cause of death

Immunohistochemistry

Human flash-frozen sections of substantia nigra (12 μm) were fixed overnight in a freshly prepared solution of 1% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4, 4°C). Sections were then processed using a modification of the method described previously by Hjelle et al. (1994). This consisted of (i) treating with 70% alcohol; (ii) pre-incubating with normal rabbit serum (20%) for 60 minutes; (iii) incubating with glutathione antiserum (gift from O.P. Ottersen) for 24 hours at a dilution of 1 : 200 and (iv) treating with biotinylated goat anti-rabbit IgG for 60 minutes and avidin/biotinylated peroxidase complex for 60 minutes. All solutions made up in 0.1 M PBS with 1% normal rabbit serum and 0.5% Triton X-100 (pH 7.4). Development was carried out by incubating with 0.05% diaminobenzidine (10 minutes) followed by the addition of 0.01% hydrogen peroxide (10 minutes) in 0.1 M TRIS-saline buffer. Further nigral immunostaining with antibodies to TH, GFAP and ricinus lectin RCA 120 for nigral dopaminergic neurons, astrocytes and microglia respectively, was also carried out according to the same method.

Statistics

The χ^2 test was used to detect possible significant differences in the intensity of mercury orange-induced fluorescence in the populations of control and PD nigral neurons sampled.

Materials

Potassium phosphate, paraformaldehyde, sucrose, mercury orange, poly-L-lysine, alcohol, xylene, DPX, Harris-haematoxylin and eosin; diaminobenzidine tetrahydrocloride, trypsin and normal horse serum were obtained from Sigma Chemical Company U.K. Toluene was obtained from Fisons U.K. Permount (Fisher Scientific, U.S.A.) was supplied by Raymond Lamb U.K. Glial fibrillary acidic protein (GFAP) antibody was obtained from DAKO U.K. The antigen anti-rabbit Ig biotinylated and streptavidin biotinylated horseradish peroxidase was supplied by Amersham U.K. Ricinus lectin was obtained from Vecta labs U.K.

Results

Mercury orange staining

Examination of nigral sections for fluorescence produced by mercury orange revealed a fibrous reticular pattern of staining in the neuropil which comprises axonal, dendritic and glial processes (Figs. 1, 2), with no fluorescence in the lumina of larger muscular vessels (Fig. 1B). Pigmented nigral neurons that were readily located by their melanin content under transmitted light microscopy revealed variable degrees of mercury orange fluorescence relative to the background neuropil. Where apparent, neuronal nuclei revealed less fluorescence and were seen in silhouette profile, defined by a surround of more intense cytoplasmic fluorescence (Figs. 1D, 2B, D). The boundary of fluorescence associated with individual neurons extended beyond the clusters of melanin granules seen under transmitted light microscopy. When melanin was only faintly present under transmitted light, fluorescence was more intense than about denser aggregates of melanin. In PD cases dense melanin clumps were often associated with less intense fluorescence than the surrounding neuropil. Additionally, a control experiment staining paraffin-

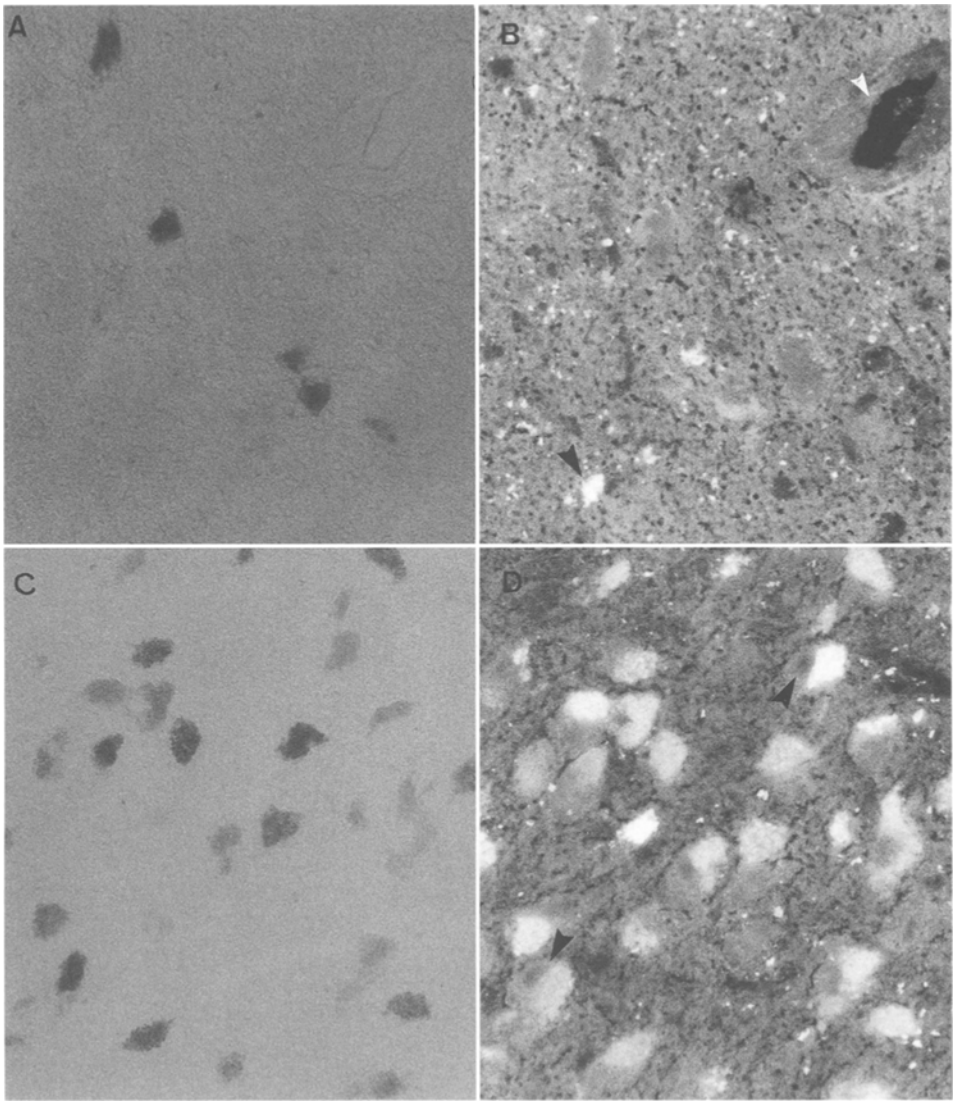

Fig. 1. Transmitted light (**A**,**C**) and fluorescent (**B**,**D**) photomicrographs of PD (**A**,**B**) and control (**C**,**D**) substantia nigras stained with mercury orange for GSH. Pigmented neurons disclosed by melanin aggregates (A,C) under transmitted light correspond to identically located structures in B and D. Neuronal nuclei show much less fluorescence than the cytoplasm (black arrow heads, D). Neurons in control nigra tend to exhibit greater fluorescence than the background neuropil (D) while PD pigmented nigral neurons exhibit fluorescence similar to or less than the background neuropil. Intensely fluorescing structure in B (black arrow head) likely represents an astro- or microglial cell. Non-fluorescing lumen of muscular blood vessel is shown in upper right corner of B (white arrow head). Original magnification 200×

embedded formalin-fixed tissue (losing GSH through processing) failed to reveal similar fluorescence in nigral neurons, suggesting that mercury orange fluorescence is associated with the cytoplasmic contents of nigral neurons rather than with melanin content per se.

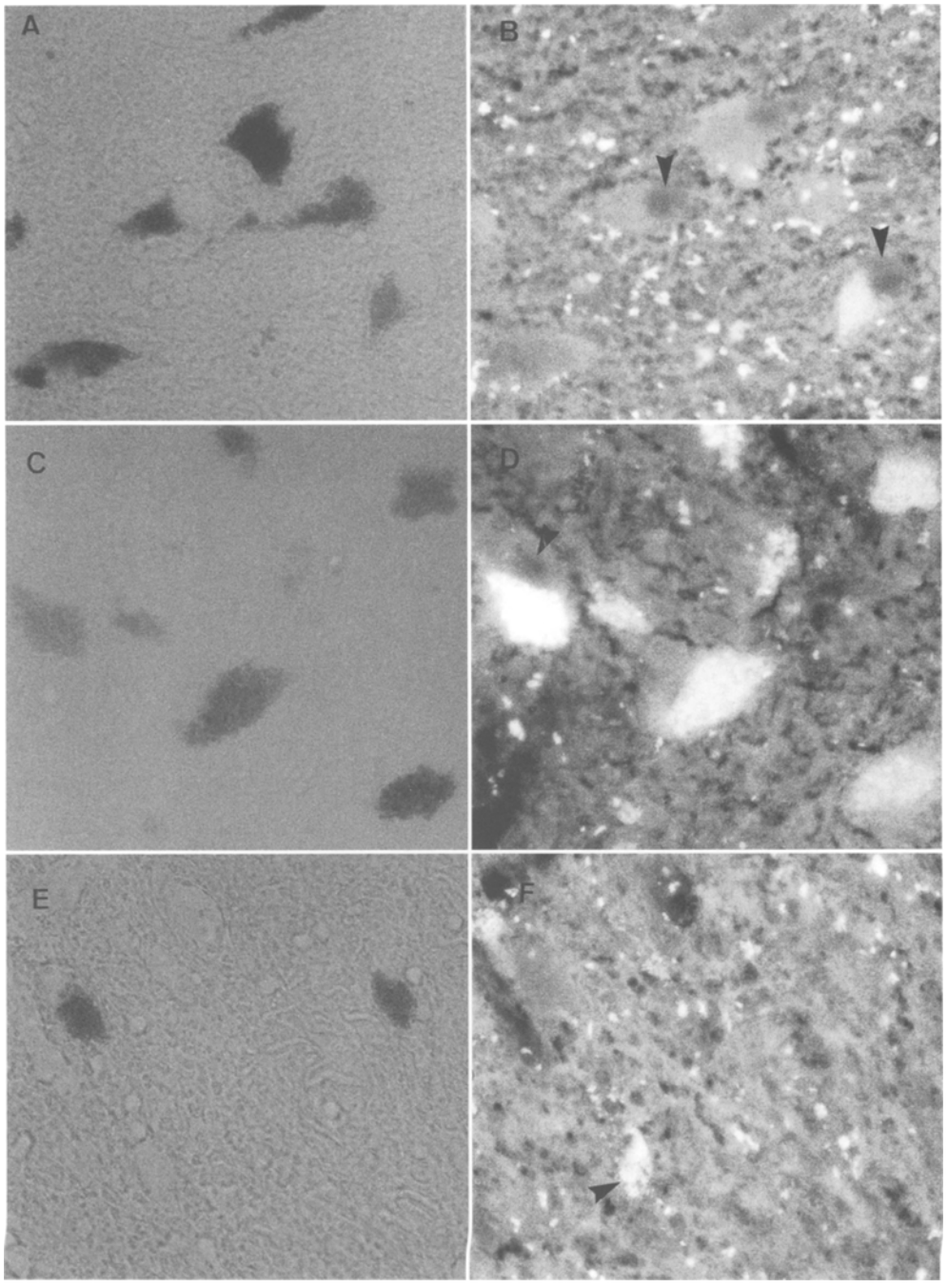

Fig. 2. Transmitted light (**A**,**C**,**E**) and fluorescent (**B**,**D**,**F**) photomicrographs of PD (**A**,**B**,**E**,**F**) and control (**C**,**D**) substantia nigra stained with mercury orange for GSH. Pigmented neurons under transmitted light in (A,C,E) correspond to identically positioned structures in (B, D, F). Control nigral neurons tend to fluorescence more intensely than the background neuropil (D). PD nigral neurons tend to fluoresce similarly to the background neuropil (B,F), in contrast to the greater fluorescence associated with control nigral neurons. Intense fluorescence likely representing astro- or microglial cell is seen in F (black arrow head) and is not associated with melanin aggregates under transmitted light in E. Neuronal nuclei reveal less intense fluorescence than the remainder of the neuronal profile (black arrow heads B,D). Original magnification 400×

Table 2. Neuronal fluorescence in substantia nigra after mercury orange staining

	+++	++	+	0	−	−−	−−−	Total
Controls								
1		18	6					24
2		18	15	2				35
3		11	5					16
4		2	1	2				5
5	6	29	11	2				48
6			5	36	1			42
Totals*	6	78	43	42	1			170
%	3.5	45.5	25.4	24.8	0.6			100
	+++	++	+	0	−	−−	−−−	Total
PD								
1			19	20				39
2			4	24				28
3			1	2	4			7
4			6	4	3			13
5					1	3		4
6		14	3	3				20
Totals*		14	33	53	8	3		111
%		12.6	29.7	47.7	7.2	2.7		100

Numbers represent melanized nigral neurons for control (C) and Parkinson's disease (PD) cases showing fluorescence after mercury orange staining equal to (0), greater than (+, ++, +++) or less than (−, −−, −−−) background neuropil when viewed at 200× magnification. *Distributions differ significantly by χ^2 test ($\alpha = 0.005$)

In control cases nigral neurons were more likely to show fluorescence greater than that of the neuropil background (Figs. 1D, 2D), while in PD tissue neurons were more prone to shown less fluorescence than the neuropil or fluorescence indistinguishable from that of the background neuropil (Figs. 1B, 2B, F).

The intensity of fluorescence in melanized nigral neurons relative to background fluorescence of the neuropil was assessed in semi-quantitative fashion. Individual neurons seen in control and PD nigra were graded blind to diagnosis on a scale of 3+ to 3− for the intensity of mercury orange fluorescence relative to the background neuropil at medium power (200X) magnification (Table 2). This method of assessment revealed that the proportion of melanin-containing cells fluorescing above background levels was greater in the control group (74.4% of all neurons) than in the PD group (42.3% of all neurons). The distributions of neuronal fluorescence relative to the fluorescence of the background neuropil for controls and PD cases were significantly different by

the χ^2 test ($\alpha = 0.005$). Thus, surviving neurons from PD cases tended to fluoresce significantly less intensely than neurons from control nigras.

Occasional intense polygonally disposed collections of mercury orange fluorescence of a granular or punctate nature present in both control and PD nigras and not associated with melanin granules suggested the profile of cells smaller than the pigmented nigral neurons and likely represent a subpopulation of glia, either astrocytes or microglia (Figs. 1B, 2F). Similarly distributed cells were seen after immunostaining with GFAP and ricinus lectin for astrocytes and microglia respectively. Scattered intense punctate fluorescence was also seen, possibly representing glial or neuronal processes or possibly undissolved particles of mercury orange.

Immunohistochemistry

A control nigra, processed without antibody, revealed no positive staining (Fig. 3A). Nigral immunostaining with an antibody specific for reduced glutathione revealed moderately intense staining of the neuropil in both control and PD cases (Figs. 3, 4). Immunostaining of the neuropil in control nigras was often appreciably more intense than in PD nigral samples, such that morphological details were obscured. Generally, in the PD cases the nigral neuropil was more likely to reveal discrete flowing axon cables and a more attenuated appearance than the control nigras. In both control and PD cases nigral neurons revealed positive staining of the cytoplasm and proximal axonal processes (Fig. 4B) with positive but slightly less intense staining of the nucleus and virtually no staining of the nucleolus (Figs. 3E, F). Some neurons of PD cases showed cytoplasmic boundaries which were more easily distinguished from melanin pigment by virtue of less intense staining, while in control cases this demarcation was more often difficult to discern. In PD cases clusters of melanin granules suggestive of intracellular neuronal pigment collections were occasionally seen against staining indistinguishable from that of the neuropil, possibly correlating with those neurons in PD nigras displaying less mercury orange fluorescence than the background neuropil. These intracellular melanin aggregates had a different appearance from the scattered melanin granules associated with pigmentary incontinence in PD which were also identifiable in these tissues.

When examining the ventral nigra about the myelinated fibres of the cerebral peduncle, or the region of the ventral tegmental area about rootlets of the oculomotor nerve, positive staining of individual axon cables could be easily discerned either in longitudinal profile or en face against non-staining myelin surrounds (Fig. 4). Within the substance of the nigra, individual coursing axon fibres could also be readily discerned, especially in PD cases. Capillary loops showed moderate staining of vessel walls (Figs. 3B, E) and in PD cases occasional Lewy body inclusions could be discerned.

The most intense immunostaining for GSH was seen in cells with the typical morphological features of astrocytes or microglia and this was detected in both control and PD nigras (Figs. 3B, C, D). Separate immunohistochemical study of formalin-fixed control and PD nigras for GFAP and ricinus lectin

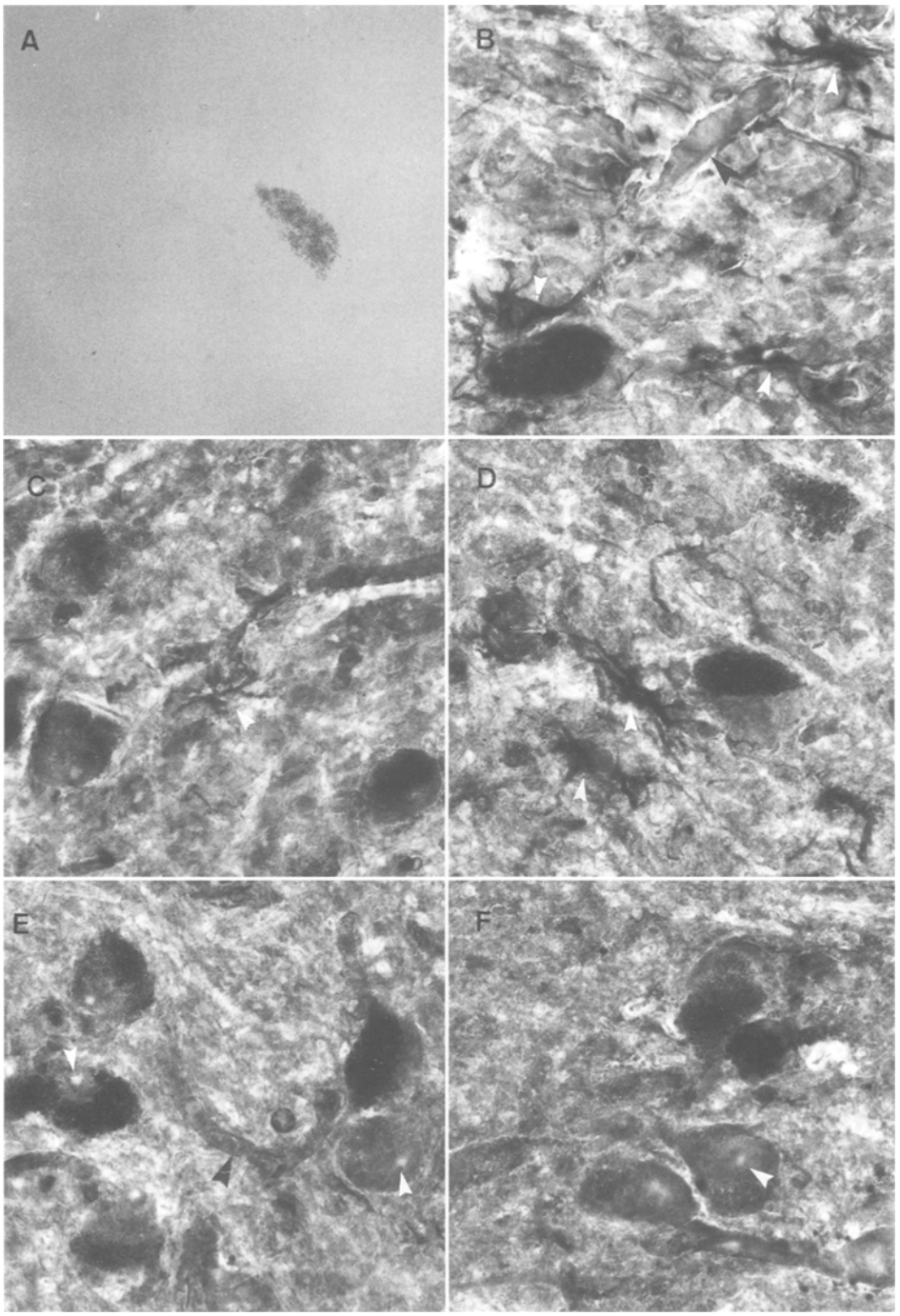

Fig. 3. Substantia nigra from control (**A**,**B**,**C**) and PD cases (**D**,**E**,**F**) stained with specific antibody for GSH (**B**–**F**) or processed without antibody present (**A**). Stained structures include neuronal cytoplasm and nuclei with staining absent from nucleoli (white arrow heads E,F); nonpigmented cells with the morphology of astrocytes or microglia show intense staining and are present in both control and PD nigras (white arrow heads B,C,D). Capillary walls are also moderately stained (balck arrow heads B,E). Neurons contain melanin granules and cytoplasmic staining tends to be less intense in some PD nigral neurons. Original magnification 400×

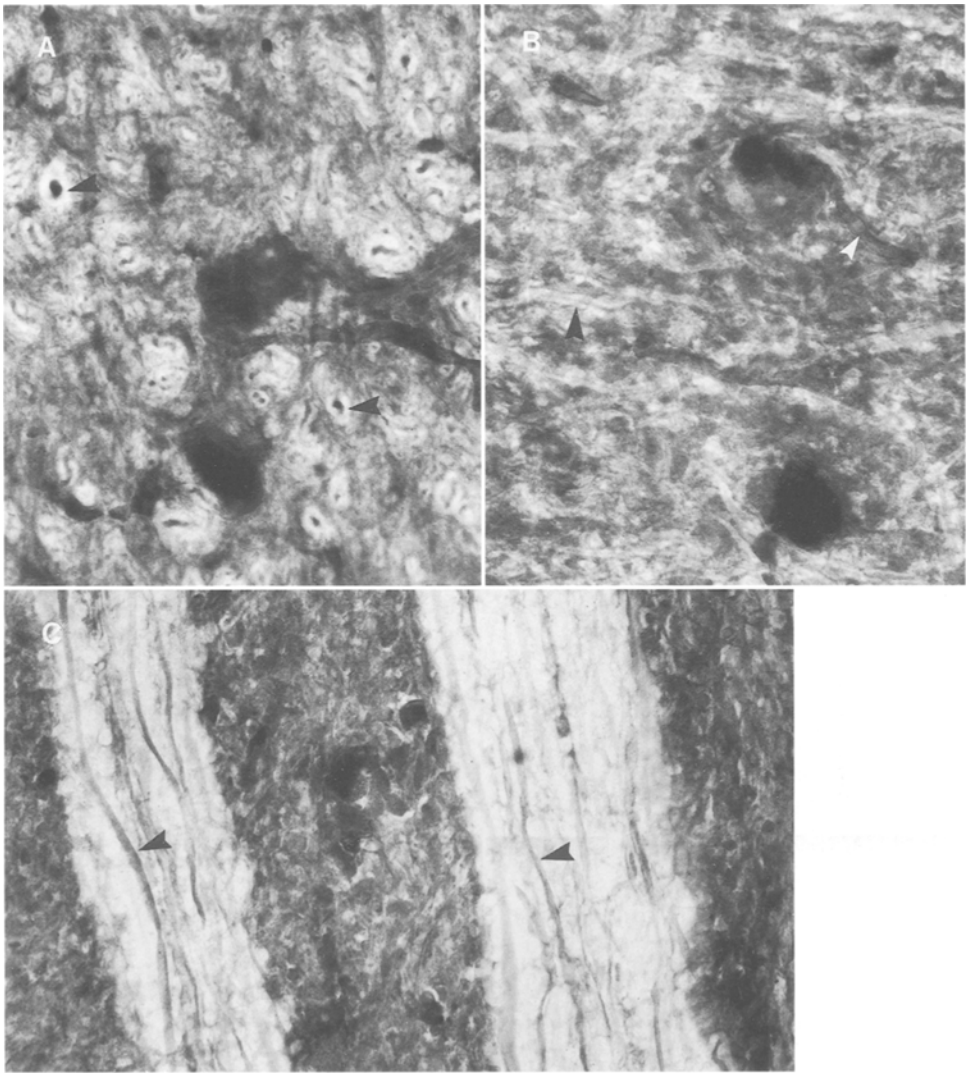

Fig. 4. Substantia nigra from control (**A**) and PD (**B**,**C**) nigras stained with antibody to GSH showing positive staining of axon fibres (black arrow heads) cut perpendicular to longitudinal axis and shown close to cerebral peduncle in the lateral nigra (A); shown coursing in many directions in the dorsal nigra (B); and comprising the myelinated fascicles of the exiting oculomotor nerve in ventral tegmental area (C). White arrow head indicates proximal axonal process in (B). Original magnification 400×

showed cells of a similar size, morphology and frequency in the nigra, helping to confirm the identity of these cells.

Discussion

The brunt of nigral pathology in PD is borne by neurons in the ventral tier of the pars compacta of the substantia nigra (Fearnley and Lees, 1991). In this study we were able to directly assess the relationship between nigral pathology and GSH depletion. Previous studies employing nigral homogenates did

not permit determination of the anatomical or cytoarchitectural distribution of GSH or its presence in specific cell types in PD. The results of the present study employing two different histological techniques suggest that in the human substantia nigra GSH is localised in both the cell body and axonodendritic processes of pigmented nigral neurons with a moderate to intense degree of background staining in the neuropil. The most intense fluorescence and immunostaining for GSH was confined to a subpopulation of glial cells with the morphological features of astrocytes or microglia. The localization of GSH to capillary loops by immunostaining also agrees with previous reports that capillary endothelial cells are enriched with the molecule (Philbert et al., 1991).

The results of this study agree with previous GSH histofluorescence studies in both rodent and monkey brain showing intense levels of background staining in the neuropil (Kirstein et al., 1991; Philbert et al., 1991; Slivka et al., 1987). In these studies employing mercury orange, significant GSH staining was found in the perikarya of several subtypes of neurons, including olfactory receptor neurones and their axons (Kirstein et al., 1991), dorsal root ganglion cells (Philbert et al., 1991), Purkinje and granule cells of the cerebellum (Slivka et al., 1987) although in most brain regions GSH content was predominantly found outside neuronal somata and confined to other elements of the neuropil, such as axon cylinders and terminals, dendritic processes, glia and the extracellular matrix (Slivka et al., 1987). The glutathione antibody employed in this study also clearly showed staining in the cytoplasm and nucleus of nigral neurons, with a conspicuous absence of staining in nucleoli, which may relate to an artefact of intense glutaraldehyde crosslinking and poor antibody penetration at this site (O.P. Ottersen, personal communication). Axonal processes were intensely stained, in agreement with previous work (Amara et al., 1994).

Earlier studies have reported a low GSH content in cultured neurons and a lack of mercury orange staining in most classes of neuronal perikarya, implying that neurons are relatively deficient in glutathione (Raps et al., 1989). However, in another report GSH levels in neuronal culture were comparable to those found in glial cultures (Bridges et al., 1991) and Pileblad et al. (1991) found that GSH content in brain stem neuronal cultures was double that of glial cultures from brain stem and cortical sties. Differing glutathione content of glia and neurons in culture may be partially artifactual, since glial cells synthesize GSH from both cysteine and cystine, whereas neurons are only able to utilise cysteine produced by glial cells (Sagara et al., 1993). Neurons would thus be unable to maintain GSH stores when chronically isolated in culture (Makar et al., 1994; Sagara et al., 1993). Cultured neurons are under oxidative stress and this may further diminish GSH content (Chau et al., 1988) Thus, the low glutathione content reported in long term neuronal cultures may not accurately depict neuronal glutathione content in vivo. It is not surprising that nigral neurons should demonstrate appreciable perikaryal GSH content, given the production of highly reactive free radical species in the course of dopamine metabolism and GSH-related enzymes have been found within neurons of the CNS (Huang and Philbert, 1995).

Significantly less intense mercury orange-induced histofluorescence of pigmented nigral neurons relative to background neuropil levels of fluorescence was found in nigral sections from PD cases when compared with controls. This indicates decreased neuronal GSH content in a subset of surviving neurons in the PD nigra. These findings support the previously published biochemical findings of an approximately 40% nigral GSH depletion in PD (Perry et al., 1982; Riederer et al., 1989; Sian et al., 1994a), and in ILBD cases (Dexter et al., 1994). The latter finding also indicates that GSH depletion in the nigra precedes nerve cell death and may thus be an early sign of neuronal dysfunction. Our results showed variation in levels of fluorescence between individual neurons in the PD and control substantia nigra, indicating subpopulations of nigral neurons of varying GSH content and perhaps a differential sucsceptibility to oxidative stress.

Whether the pronounced general decrease in neuronal GSH content in the PD nigra represents increased consumption or decreased production remains unclear. There did not appear to be a relation between the intensity of mercury orange fluorescence and post mortem interval for individual cases or for the control and PD groups studied, with the control group having a longer mean time to brain removal (21.25 hours versus 17.2 hours for PD cases), making it unlikely that the greater fluorescence observed in the control group was due to an artifact of post mortem delay, since less GSH would be expected with a longer interval between death and tissue preservation.

Immunohistochemical staining of both control and PD nigra for GSH showed intense staining in cells with the morphological features of astrocytes or microglia as confirmed by GFAP and ricinus lectin immuno-staining. These cells were sparsely represented and thus could not be reliably quantitated in the tissues. The immunostaining of these cells may correlate with the discrete polygonal clusters of intense mercury orange-evoked fluorescence observed in control and PD specimens but which were not readily identifiably using the fluorescence technique. Subpopulations of glial cells have differing rates of glutathione turnover and the intense expression of GSH may indicate a functional adaptation of these cells in the substantia nigra where the native metabolism of dopamine occasions significant oxidative stress (Jenner et al., 1992a; Makar et al., 1994). Increased microglia and astrocytes are found in the nigra in PD (Damier et al., 1993; McGeer et al., 1993). Thus, glial handling of glutathione may have some relevance in either containing or inciting oxidative stress in PD. The report of glutathione peroxidase-immunoreactive cells with the morphological attributes of astrocytes surrounding surviving nigral neurons in the PD nigra supports this notion (Damier et al., 1993; McGeer et al., 1988).

Previous reports of both mercury orange and specific antibody staining for GSH have shown that in most brain regions it is strongly localized in axonodendritic processes (Amara et al., 1994; Philbert et al., 1991; Slivka et al., 1987). This was also the case in the human nigra with both techniques in the present investigation. Philbert et al. (1991) showed a moderate degree of staining in white matter tracts and noted the presence of glutathione in both the myelin sheath and axon cylinder, though employing immunostaining we

clearly show that intense staining of axon cylinders is accompanied by an absence of staining in the myelin sheath. Physiological studies utilising rat brain slices suggest that endogenous glutathione is released from axonal terminals and may play a role in synaptic transmission, quite apart from its role as an antioxidant (Zängerle et al., 1992). In light of our present results and given the large volume of the pars compacta of the substantia nigra occupied by neuronal somata and their axonodendritic processes, it is highly likely that the reported decrease in nigral GSH in PD and ILBD directly reflects a disturbance of GSH content in nigral dopaminergic neurons. Similarly, the normal levels of oxidized glutathione (GSSG) reported for the PD nigra suggests an increased level of oxidized glutathione per surviving nerve cell.

The reduction in nigral GSH content in PD reflects impairment of a major defense system against oxidative stress. It emphasizes the potential importance of GSH in the pathogenesis of PD, particularly as this is the only index of oxidative stress altered in the prodromal condition ILBD (Dexter et al., 1994). Impairment of the GSH defence system would compromise the ability of the brain to eliminate hydrogen peroxide, resulting in secondary mitochondrial dysfunction (Di Monte et al., 1992; Jenner et al., 1992a, b; Perry and Yong, 1986). Mitochondria produce hydrogen peroxide, which is detoxified by GSH-dependent mechanisms and a GSH deficit has been shown to exacerbate neuronal damage by impairing mitochondrial function independently of the presence of oxidative stress (Jain et al., 1991; Meister, 1995). Brain aging itself results in decreased GSH and altered mitochondrial function and in aged rats the induction of oxidative stress results in GSH depletion and impaired mitochondrial function (Benzi et al., 1992) which may resemble the situation in PD, with oxidative stress and altered mitochondrial activity reflected in defects in complex I of the electron transport chain (Schapira et al., 1990). Mitochondria obtain GSH from the cytosolic pool and contain about 10% of cellular GSH (Huang and Philbert, 1995). Thus, any process reulting in a depletion of cytosolic GSH might reduce mitochondrial GSH stores and critically impair cellular energy production.

GSH depletion may also render the brain more vulnerable to exogenous toxins. Midbrain dopaminergic neurones in the rat are more vulnerable to the toxic effects of 6-hydroxydopamine after reduction of GSH stores by L-buthionine sulfoximine (Pileblad et al., 1989). The possible role of chronic L-DOPA administration in either exacerbating or attenuating GSH depletion remains controversial (Han et al., 1996). GSH is active in keeping both α-tocopherol and ascorbate in the reduced state (Meister, 1992) and thus a nigral GSH deficit may also exert deleterious secondary effects in the protective capability of other antioxidants such as vitamin E and vitamin C, even though the nigral content of these two antioxidants is not altered in PD (Jenner et al., 1992a; Wefers and Sies, 1988).

Although the distribution of glutathione in brain may be a major determinant of susceptibility to toxic chemical species such as free radicals (Philbert et al., 1991), nigral GSH reduction in PD may also be related to a generalized defect in endogenous sulphur metabolism, rendering the individual more susceptible to toxic xenobiotics; plasma cysteine: sulphur ratios have been

reported to be elevated in PD and patients also display imparied sulfation of xenobiotics (Heafield et al., 1990).

This histological study confirms the depletion of GSH in the substantia nigra in PD and for the first time demonstrates an appreciable presence of GSH in human nigral neurons and a specific depletion of neuronal GSH in the PD nigra. These results support the notion that oxidative stress may underlie the progressive loss of nigrostriatal dopaminergic neurons in PD. These conclusions also suggest that therapeutic measures designed to contain oxidative stress and maintain the integrity of antioxidant defenses such as GSH may be of importance in slowing or halting the progression of neuronal degeneration in PD and may be of benefit in the earliest stages of the disorder (Dexter et al., 1991; Jenner et al., 1992a,b).

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