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Short communication

The distribution of copper, zinc- and manganese-superoxide dismutase, and glutathione peroxidase messenger ribonucleic acid in rat basal ganglia

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Abstract

Oxidative stress may contribute to the progression of Parkinson's disease, and while the status of antioxidant enzymes is thus important, little data on their regional distribution in basal ganglia exist. We now report on the distribution and levels of messenger ribonucleic acid (m-RNA) for the antioxidant enzymes copper, zinc-superoxide dismutase (Cu,Zn-SOD), manganese-superoxide dismutase (Mn-SOD), and glutathione peroxidase in rat basal ganglia using *in situ* hybridisation histochemistry with complementary deoxyribonucleic acid probes specific for these enzymes. The m-RNA for Cu,Zn-SOD, Mn-SOD, and glutathione peroxidase was expressed throughout basal ganglia. Levels of m-RNA were significantly higher in substantia nigra pars compacta than in all other regions of basal ganglia for both Cu,Zn-SOD (53–62%, $P < 0.001$) and Mn-SOD (37–45%, $P < 0.05$). Mn-SOD m-RNA levels were also significantly higher in SN pars reticulata than in the nucleus accumbens (10%, $P < 0.05$) and striatum (12%, $P < 0.01$). In contrast, glutathione peroxidase m-RNA levels were only significantly higher in SN pars compacta when compared with SN pars reticulata (23%, $P < 0.05$), and in the striatum when compared with the nucleus accumbens (21%, $P < 0.05$). The data suggest that SN pars compacta may be vulnerable to oxidative stress and thus dependent on the high antioxidant capacity provided by these cytoprotective enzymes. In conclusion, this study demonstrates the relative distribution of antioxidant enzymes in rat basal ganglia and forms the basis for further study in rodent models of Parkinson's disease.

Keywords: Antioxidant enzymes; Distribution; m-RNA; Basal ganglia; Parkinson's disease; Oxidative stress

1. Introduction

Progressive neurodegeneration of substantia nigra (SN) characterises Parkinson's disease (PD), although the pathological process responsible remains unknown. Loss of nigral dopaminergic neurones, however, has been associated with oxidative stress [16]. Post-mortem studies have demonstrated lipid peroxidation [11], protein [2] and deoxyribonucleic acid [3] oxidative damage, increased iron levels [12,33], and inhibition of mitochondrial function [35] in SN of patients with PD.

The status of the antioxidant defence system is, therefore, of great importance in PD. Whilst nigral levels of the antioxidant Vitamins C and E are unchanged, those of both total and reduced glutathione are decreased [33,36]. Reported changes in activities of the antioxidant enzymes, however, are conflicting. For example, total SOD enzyme activity in PD has been reported to be increased in the putamen and SN by Marttila *et al*. [25], but unchanged in SN by Saggu *et al*. [34]. As regards Cu,Zn-SOD (EC 1.15.1.1), studies report unchanged protein levels [26] and enzyme activity [34] in SN, and unchanged gene expression in the putamen and SN [31]. In contrast, Marttila *et al*. [25] found that the enzyme activity of Mn-SOD (EC 1.15.1.1) in Parkinsonian SN was normal, whilst Poirier *et al*. [31] found that it was increased in the

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Abbreviations: SN, substantia nigra; PD, Parkinson's disease; SOD, superoxide dismutase; Cu,Zn-SOD, copper, zinc-superoxide dismutase; Mn-SOD, manganese superoxide dismutase; GP, globus pallidus; m-RNA, messenger ribonucleic acid; ISHH, *in situ* hybridisation histochemistry; SNpc, substantia nigra pars compacta; CP, striatum; SNpr, substantia nigra pars reticulata; cDNA, complementary deoxyribonucleic acid; NA, nucleus accumbens.

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putamen and SN. Immunostaining studies in PD also conflict: increased levels of Mn-SOD protein have been found in the dendritic process of melanised nigral neurones [41], as well as decreased protein levels in the putamen and SN [31]. Decreased m-RNA levels of Mn-SOD have also been reported in Parkinsonian putamen and SN [31]. There is also some confusion with glutathione peroxidase (EC 1.11.1.9) in PD. Early studies report decreased glutathione peroxidase activity in the GP, putamen, and SN [4,17,18], but later studies show normal activity in these brain areas [25,37]. Such contradictions may be due to the different techniques used in each study, including enzyme assays, immunohistochemistry, and Northern blot analysis [17,31,34].

Little information exists on the levels or distribution of Cu,Zn-SOD, Mn-SOD, or glutathione peroxidase m-RNA in human basal ganglia and few studies have used ISHH to investigate how the pathology of PD may affect these enzymes at the molecular level. Distribution studies for SOD have focused mainly on enzyme activity or protein localisation in non-basal ganglia areas and have been restricted largely either to mouse or human tissues [1,10]. Enzymatic studies in human tissue report high SOD activity in SN [24,25,26], whilst immunohistochemical studies show Cu,Zn-SOD protein in SN [7,24,25]. Bergeron *et al.* [5], however, have used ISHH to identify Cu,Zn-SOD m-RNA in the cytoplasm and proximal processes of SNpc, while others demonstrated Cu,Zn-SOD m-RNA expression in the neuromelanin-pigmented neurones and dopaminergic neurones of SNpc that are susceptible to neurodegeneration in PD [7,43]. Similarly, high levels of Mn-SOD protein are found in the putamen and SN of human basal ganglia; it is expressed in neuronal but not glial cells in SN, in both neuronal and glial cells in the caudate and putamen, and only in neurones of the GP [42]. Meanwhile, high glutathione peroxidase-like immunoreactivity occurs in human glial cells that surround dopaminergic neurones resistant to neurodegenerative damage in PD, but levels are lower in areas susceptible to damage, such as SNpc [9].

In rats, a species commonly used as a model to study lesion-related changes in PD, studies have demonstrated high total SOD activity, particularly in rodent CP and SN [22,27,39]. The enzyme activity of SOD corresponds to protein distribution in rodent basal ganglia [38], particularly with respect to Mn-SOD protein, which occurs in the large neurones of SNpr and, to a lesser extent, SNpc [23]. In addition, high levels of Cu,Zn-SOD protein are expressed in the cell bodies, dendrites, and axons of mouse motor neurones [28]. Similarly, glutathione peroxidase activity is high in rat CP and SN [6] and its protein is distributed widely in both neuronal and glial cells of murine basal ganglia, especially the caudate nucleus, and to a lesser extent in SNpc [40].

Nevertheless, other than our preliminary study on relative distribution of Cu,Zn-SOD and Mn-SOD in adult rat brain [21], ISHH has not been used to determine the distribution and levels of antioxidant enzyme m-RNA in rat basal ganglia. We now report on the relative distribution and levels of Cu,Zn-SOD, Mn-SOD, and glutathione peroxidase m-RNA in rat basal ganglia using specific cDNA probes and ISHH as a prelude to extending these studies to the 6-hydroxydopamine-lesioned rat model of PD and subsequently to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primate, and finally to PD itself.

2. Materials and methods

All materials were obtained from Sigma Chemical Company unless otherwise stated. Adult male Wistar rats (200–250 g; Bantin & Kingman) were terminally anaesthetised using sodium pentobarbitone (150 mg/kg IP; Rhone Merieux) and decapitated. As described previously [20], brains were removed, snap-frozen in isopentane (-40°; BDH), and every 16th, contiguous, coronal section (12 μm) was collected from each brain between the anterior co-ordinates (mm from bregma) +2.70 to +0.48 (NA), +2.20 to −3.30 (CP), −0.80 to +2.80 (GP), and −4.80 to −6.04 (SNpc and SNpr) [29] onto sterile, chrome-alum/gelatin-coated slides (BDH) and stored at -70°. Thawed brain sections were pre-hybridised by fixing in 0.1 M phosphate buffered saline (0.9% sodium chloride containing 1.2 mM potassium hydrogen phosphate and 5.6 mM disodium hydrogen phosphate dodecahydrate, pH 7.4; BDH) containing 4% *para*-formaldehyde (BDH) for 15 min at 25°. Sections were washed in phosphate buffered saline, dehydrated in ethanol (70–100%) (BDH), air-dried, and used for hybridisation immediately.

Oligodeoxynucleotide (single-stranded cDNA) probes were custom synthesised (Pharmacia) for the following sequences: 5′-cc agt ctt tgt act ttc ttc att tcc acc ttt gcc caa gtc atc-3′ (Cu,Zn-SOD); 5′-tga tct gcg cgt taa tgt gcg gct cca gcg cgc cat agt c-3′ (Mn-SOD); and 5′-tat cgg gtt cga tgt cga tgg tgc gaa agc gcc tgc tgt atc-3′ (glutathione peroxidase), which showed 100% identity to bases 369–412 [32], 111–150 [14], and 878–919 [15] in rat, respectively. Based on established methodology [20], probes (100 ng) were labelled with α-[^{35}S]dATP deoxyadenosine triphosphate (NEN DuPont) using 'One-Phor-All' reaction buffer and terminal deoxynucleotidyl transferase (Pharmacia). Following incubation at 37° for 30 min, the reaction was terminated using 75 μL Tris–EDTA buffer [1 M Tris (Boehringer Manheim) + 0.5 M EDTA; pH 7.6]. Labelled probe fractions (100–300 × 10^3 dpm/μL) were collected using a NICK^{TM} separating column (Pharmacia) and Tris–EDTA buffer (400 μL), pooled (approximate probe-labelling was 400, 200, and 150 × 10^3 dpm/μL for Cu,Zn-SOD, Mn-SOD, and glutathione peroxidase, respectively), and dithiothreitol (1 M dissolved in 0.01 M sodium acetate, pH 5.2) (1 μL/100 μL probe) was added. This probe solution (2 ng/100 μL) and β-mercaptoethanol (3 μL/100 μL) were

added to hybridisation buffer consisting of 50% de-ionised and filtered formamide (Fluka), saline sodium citrate (0.75 M sodium chloride and 0.075 M sodium citrate; pH 7.0), Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.5 mg/mL sheared salmon sperm deoxyribonucleic acid, and 10% (w/v) dextran sulphate, the final amount of radiolabelled probe in hybridisation buffer being approximately 4×10^3 dpm/μL for all the three probes. Pre-hybridised sections were incubated for 18 hr at 37° with 250 μL (approximately 5 ng labelled probe per slide) hybridisation buffer.

Brain sections were post-hybridised by rinsing in distilled water and in saline sodium citrate (0.15 M sodium chloride and 0.015 M sodium citrate; pH 7.0) at 25°, and then in three consecutive baths of saline sodium citrate containing β-mercaptoethanol (1/100 μL) at 55° for 30 min. Sections were rinsed in distilled water at 25°, dehydrated in 70% alcohol containing 300 mM ammonium acetate (BDH), dried, and apposed with commercial [^{14}C]-methylmethacrylate autoradiographic standards (Amersham) for 2–4 weeks against BioMax film (Kodak). The film was developed and fixed in D-19 developer and A3000 containing HX-40 hardener, respectively (Kodak).

The specificity of probe hybridisation was demonstrated by eliminating the hybridisation signal either with a 50-fold excess of unlabelled probe in the hybridisation buffer, or by pre-treating brain sections with ribonuclease (Type III-A; 100 μg/mL in 10 mM Tris–HCl; pH 8.0) containing sodium chloride (0.5 M) for 30 min at 37° after which sections were washed in Tris–HCl (10 mM; pH 8.0), sterile water and dehydrated in ethanol, air-dried, and hybridised as described above.

Autoradiograms were analysed by computer densitometry using MCID-M1 image analysis software (Imaging Research). The signal represented the amount of cDNA probe-target m-RNA hybrids present in the tissue and results were expressed as mean density (nCi/mg tissue dry weight). The hybridisation signal was completely abolished (as described above) for both SOD probes, while some glutathione peroxidase non-specific hybridisation was detected and thus subtracted from total hybridisation. All subsequent results are for specific probe hybridisation (equivalent to m-RNA levels) in basal ganglia areas studied. Specific hybridisation values obtained from both sides of each brain were pooled prior to determining a mean value per brain area in each rat ($n = 6$ rats per determination). Data was analysed using repeated measures ANOVA followed by paired Student's *t*-test for significance.

3. Results and discussion

This is the first study using ISHH to investigate the distribution of Cu,Zn-SOD, Mn-SOD, and glutathione peroxidase m-RNA in rat basal ganglia. All three antioxidant enzymes were expressed throughout the basal ganglia (Figs. 1 and 2). The distribution of Cu,Zn-SOD m-RNA in basal ganglia in this study (Fig. 2) complements data from previous enzymatic [22,24–26,39], immunohistochemical [28,38,43] and ISHH [5] studies. While the mean density (nCi/mg) of Cu,Zn-SOD m-RNA was approximately the same in most basal ganglia areas investigated, it was significantly higher only in SNpc compared with all other areas (53–62%, $P < 0.001$) (Fig. 2).

Mn-SOD m-RNA was also detected in all regions of basal ganglia investigated (Figs. 1 and 2), complementing data from previous studies [25,31,41,42]. As with Cu,Zn-SOD, the mean density (nCi/mg) of Mn-SOD m-RNA was significantly higher (37–45%, $P < 0.05$) in SNpc than in all other areas (Fig. 2). Moreover, levels were significantly higher in SNpr compared with both NA (10%, $P < 0.05$) and CP (12%, $P < 0.01$) (Fig. 2).

Similarly, glutathione peroxidase m-RNA was found in all regions of basal ganglia investigated, although its distribution was far more diffuse and less well-defined according to specific structures (Figs. 1 and 2). This wide distribution of glutathione peroxidase m-RNA throughout rat basal ganglia correlates well with that of its protein in human [9] and murine [40] brain tissue, and enzyme activity in rodent brain tissue [6]. Glutathione peroxidase has been found in glial cells, particularly the astrocytes [30], in those regions of human brain with dopaminergic neurones resistant to neurodegenerative damage [9]. Glutathione peroxidase-positive glial cells are less abundant, however, in dopaminergic areas susceptible to PD pathology, and this low density in human SNpc may explain its susceptibility to damage in PD [9]. While no specific areas of expression could be distinguished easily, the CP and SNpc regions appeared to express higher levels of glutathione peroxidase m-RNA than other basal ganglia areas (Fig. 2). This was only significant, however, when comparing CP with NA (21%, $P < 0.05$) and SNpc with SNpr (23%, $P < 0.05$).

Notably, while levels of Cu,Zn-SOD m-RNA appear higher throughout basal ganglia than those of both Mn-SOD and glutathione peroxidase, such direct comparisons cannot be verified here using ISHH. Although, the probes were designed to have the maximal glycine:cytosine content to ensure effective hybridisation, the content does vary between our three probes. This, together with the fact that the three-dimensional structure of m-RNA for each enzyme is different, may affect probe sensitivity and result in a different affinity for its corresponding m-RNA.

Interestingly, here we have shown the m-RNA levels of all three enzymes to be highest in SNpc, suggesting that this area may be vulnerable to free radical toxicity and thus dependent upon the high antioxidant capacity provided by these enzymes. Indeed, in normal human brain, the increased protein oxidation found in SNpc suggests that oxidative damage is higher in the dopaminergic neurones

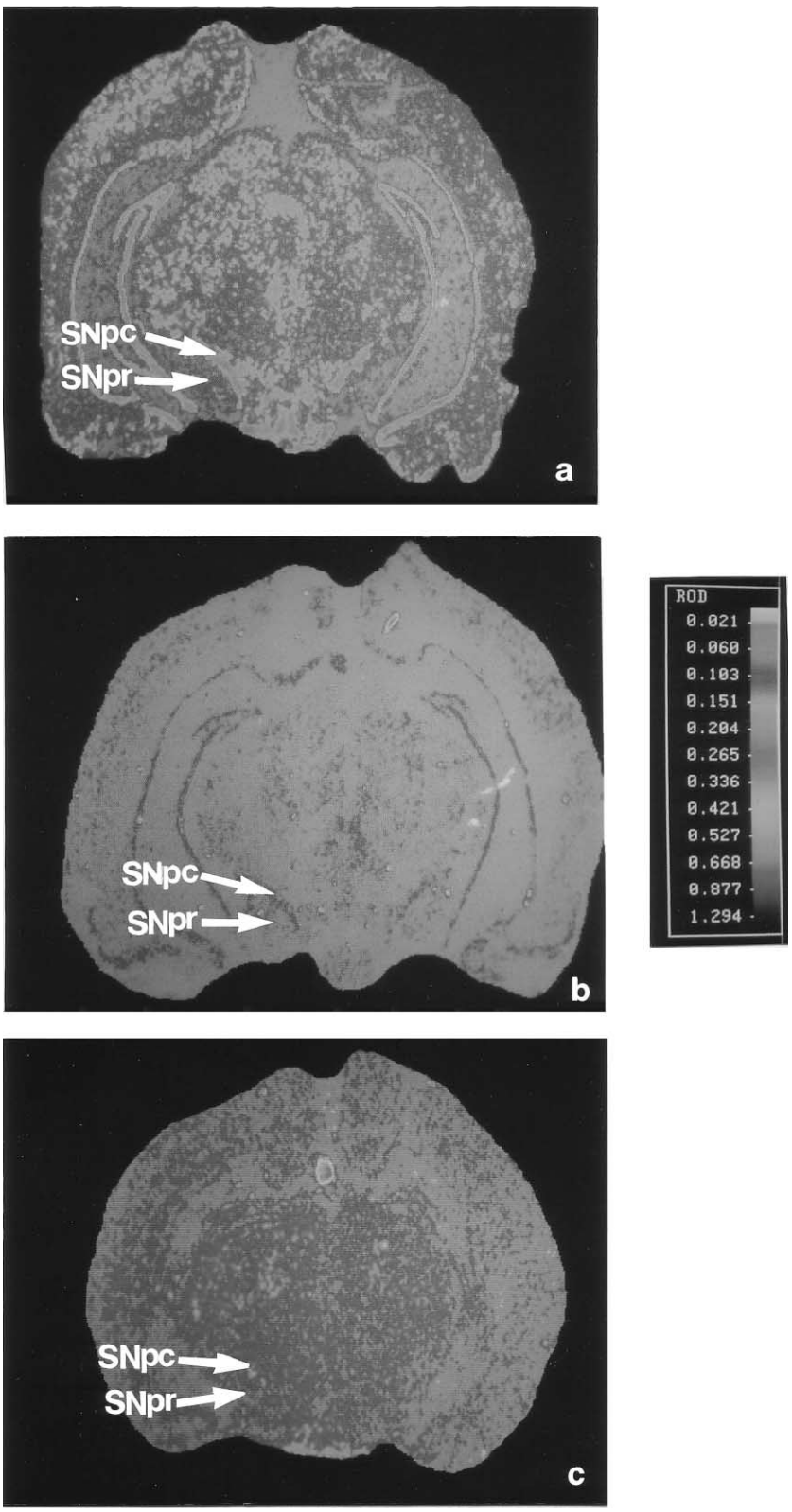

Fig. 1. Photographs of pseudo-color digitised autoradiographic images showing the hybridisation of the ^{35}S-labelled (a) Cu,Zn-SOD; (b) Mn-SOD and (c) glutathione peroxidase probes to SNpc and SNpr in coronal sections (12 μm) from normal adult rat brain. Photographs represent typical sections collected between the anterior co-ordinates (mm from bregma) −4.80 to −6.04 for SN. The calibration bar for all images is shown as relative optical density (ROD) on the right.

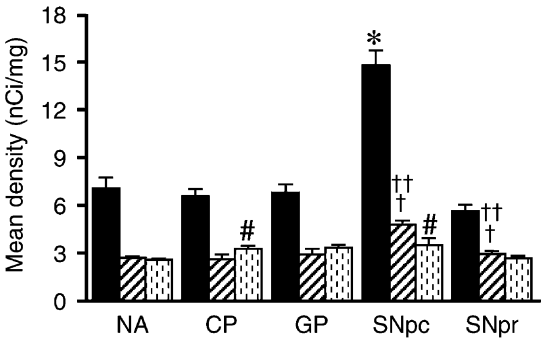

Fig. 2. The distribution of (■) Cu,Zn-SOD, (▨) Mn-SOD, and (▦) glutathione peroxidase m-RNA (nCi/mg) in the NA, CP, GP, SNpc, and SNpr of basal ganglia in rat brain, where $n = 6$ rats for each area. Mean control values (0 nCi/mg tissue for Cu,Zn-SOD and Mn-SOD, $n = 4$; 1.48 nCi/mg ± 0.21 SEM, $n = 4$ for glutathione peroxidase) were subtracted from each value prior to mean calculation. (∗) $P < 0.001$ when comparing Cu,Zn-SOD m-RNA levels in SNpc with NA, CP, GP, and SNpr; (†) $P < 0.01$ when comparing Mn-SOD m-RNA levels in SNpc with NA, CP, GP, and in SNpr with CP; (††) $P < 0.05$ when comparing Mn-SOD m-RNA levels in SNpr with NA, and in SNpc with SNpr; (#) $P < 0.05$ when comparing glutathione peroxidase m-RNA levels in CP with NA, and in SNpc with SNpr using repeated measures ANOVA and paired Student's *t*-test for significance.

[13] and that these neurones rely on antioxidant enzymes for protection against free radical attack. Notably, over-expression of Cu,Zn-SOD in transgenic rats has been shown to protect rat striatum against neuronal damage post-cerebral ischaemia and reperfusion [8], while mice deficient in cellular glutathione peroxidase are more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, the neurotoxin associated with PD [19].

In summary, this present study forms an important foundation for our subsequent studies in the 6-hydroxydopamine-treated rodent model of PD to determine the role of the antioxidant enzymes in PD. It also clearly demonstrates the relative distribution of these enzymes and suggests they play an important antioxidant role in basal ganglia.

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