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ORIGINAL PAPER

Alteration in Glutathione Content and Associated Enzyme Activities in the Synaptic Terminals but not in the Non-synaptic Mitochondria from the Frontal Cortex of Parkinson's Disease Brains

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Abstract Altered redox dynamics contribute to physiological aging and Parkinson's disease (PD). This is reflected in the substantia nigra (SN) of PD patients as lowered antioxidant levels and elevated oxidative damage. Contrary to this observation, we previously reported that non-SN regions such as caudate nucleus and frontal cortex (FC) exhibited elevated antioxidants and lowered mitochondrial and oxidative damage indicating constitutive protective mechanisms in PD brains. To investigate whether the subcellular distribution of antioxidants could contribute to these protective effects, we examined the distribution of antioxidant/oxidant markers in the neuropil fractions [synaptosomes, non-synaptic mitochondria and cytosol] of FC from PD (n = 9) and controls (n = 8). In the control FC, all the antioxidant activities [Superoxide dismutase (SOD), glutathione (GSH), GSH peroxidase (GPx), GSH-S-transferase (GST)] except glutathione reductase (GR) were the highest in cytosol, but several fold lower in mitochondria and much lower in synaptosomes. However, FC synaptosomes from PD brains had significantly higher levels of GSH ($p = 0.01$) and related enzymes [GPx ($p = 0.02$), GR ($p = 0.06$), GST ($p = 0.0001$)] compared to controls. Conversely, mitochondria from the FC of PD cases displayed elevated SOD activity ($p = 0.02$) while the GSH and related enzymes were relatively unaltered. These changes in the neuropil fractions were associated with unchanged or lowered oxidative damage. Further, the mitochondrial content in the synaptosomes of both PD and control brains was ≥five-fold lower compared to the non-synaptic mitochondrial fraction. Altered distribution of oxidant/antioxidant markers in the neuropil fractions of the human brain during aging and PD has implications for (1) degenerative and protective mechanisms (2) distinct antioxidant mechanisms in synaptic terminals compared to other compartments.

Keywords Parkinson's disease · Frontal cortex · Neuropil · Synaptosomes · Mitochondria · Oxidative stress · Glutathione

Abbreviations

PD	Parkinson's disease
SN	Substantianigra
FC	Frontal cortex
GSH	Glutathione reduced
PMI	Postmortem interval
3-NT	3-nitrotyrosine
GFAP	Glial fibrillary acidic protein
SOD	Superoxide Dismutase
GST	Glutathione-s-transferase
GR	Glutathione reductase
GP	Xglutathione peroxidase
ns mito	Non-synaptic mitochondria
syn mito	Synaptic mitochondria
cyto	Cytosol
CS	Citrate synthase
MDH	Malate dehydrogenase
SDH	Succinate dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Introduction

Parkinson's disease (PD) is a neurodegenerative disease clinically manifesting with movement disorders [1]. Oxidative stress and mitochondrial dysfunction contribute to the loss of dopaminergic neurons in the substantia nigra (SN) in PD [2–5]. Oxidative stress in PD manifests as lowered glutathione (GSH) [6], increased iron content and oxidative damage of cellular biomolecules [7–14], probably leading to mitochondrial dysfunction [15, 16].

Although neurodegenerative events affect specific neuroanatomical areas such as SN, histopathological staging in PD brains revealed the initial pathology in the brainstem nuclei including the SN, extending to involve the neocortex in the late-stage of the disease [17]. To investigate whether the redox dynamics seen in the SN is evident in other neuroanatomical regions, we examined the status of oxidant and antioxidant markers in the striatum (caudate nucleus and putamen) and frontal cortex (FC) in human PD brains [18]. We observed that while SN showed higher oxidative damage, the non-SN regions displayed relatively lower oxidative and mitochondrial damage. This relative sparing of some of these areas was associated with 3–5 fold higher GSH content and elevated astrocytic proliferation [(indicated by higher expression of glial fibrillary acidic protein (GFAP)] [18].

Different sub-cellular compartments of the human brain display varied response to distinct physiological and pathological stimuli, with the synaptic terminals being more susceptible to damage. Postsynaptic regions are exposed to higher calcium influx and oxidative stress due to activated glutamate receptors which might make them more vulnerable to neurodegeneration [19]. Synaptic mitochondria are more sensitive to oxidative damage compared to non-synaptic mitochondria [20–23]. Interestingly, oxidative stress in the cytosol [4, 24] could ultimately lead to synaptic dysfunction and neurodegeneration [5, 25, 26]. Similarly, oxidative damage to nuclear DNA can influence the status of synaptic proteins [6, 12, 27, 28]. The status of the cytosolic GSH significantly influences plasma membrane function at the synaptic terminals [13, 29]. These data not only highlight that synaptic terminals are the initial targets for damage and degeneration, but also suggest that one sub-cellular compartment can influence the other. Secondly, analysis of specific markers in total extracts might not reflect the relative differential distribution in individual sub-cellular compartments. Thirdly, analysis of isolated synaptic terminals will provide neuron-specific information unlike the study on total brain extracts which might represent both neuronal and non-neuronal cell populations including vascular elements.

Since the FC in PD brains showed relatively lower oxidative damage and significantly high levels of GSH [18], the study of relative distribution of oxidant markers and antioxidant activities in different sub-cellular compartments can provide a mechanistic understanding of the neuronal function and protection of this neuroanatomical region against oxidative damage in PD. Towards this objective, in the current study, we examined the status of different oxidant and antioxidant markers in synaptosomes, non-synaptic mitochondria and cytosol in the human FC from cases of PD ($n = 9$) compared to age-matched control brains ($n = 8$).

Materials and Methods

All chemicals used were of analytical grade. Bulk chemicals were obtained from Merck (Whitehouse Station, NJ, USA) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, Maharashtra, India). Nitrocellulose membrane from Millipore (Billerica, MA, U.S.A.), mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) (clone GA-5) from Biogenex (San Ramon, CA, U.S.A.), horseradish peroxidase conjugated secondary antibodies from Bangalore Genei (Bangalore, Karnataka, India), anti-dinitrophenyl (DNP), rabbit polyclonal anti-β-actin and anti-3-nitrotyrosine antibodies and protease inhibitor cocktail from Sigma (Eugene, OR, U.S.A.) were obtained.

Human Tissue Samples

Brain samples from cases satisfying neurological criteria (based on the grading system of Hoehn and Yahr [1]) and neuropathological parameters (based on histology and immunohistochemistry as described previously [18]) for PD ($n = 9$) were obtained from the Human Brain Tissue Repository (HBTR), Department of Neuropathology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore.

Non-PD control brains ($n = 8$) were from age-matched normal human subjects who succumbed to road traffic accidents (non-alcoholics, non-diabetics, not on any medication and without any known neurological or psychiatric disorders). Both diseased and control brain tissues were collected with informed consent from the close relatives of the deceased and the Institutional Ethics Committee approved the study protocol. Demographic and clinical details of all the subjects were recorded. Within 1 h of death, the body was transferred to a refrigerator maintained at 2–4 °C with a recorder and uninterrupted power supply. Following autopsy, the brains were sliced coronally and kept flat on salt-ice mixture (−15 to −18 °C) during dissection and then transferred into plastic zip lock bags and stored at −80 °C in cryo-resistant plastic boxes. The procedure of dissection of the fresh brains took 30–45 min and the brain slices were transferred immediately

Table 1 Details of brain samples collected from PD cases and controls (See methods)

Sample no.	Age (years)/sex	Post mortem interval (hours)	Hoehn and Yahr scale (1)
PD			
1	35/M	2	Parkinsonism plus
2	48/F	12.33	Stage I
3	56/M	1.5	Stage I
4	62/M	2.66	Stage IV
5	65/M	6.5	Parkinsonism plus
6	68/M	15	Parkinsonism plus
7	75/M	9.5	Parkinsonism plus
8	76/M	2	Stage III
9	77/M	3.25	Parkinsonism plus
Control			
1	35/M	7.5	
2	50/M	15	
3	55/M	7	
4	60/M	20	
5	65/M	22	
6	70/M	19.5	
7	77/F	13.6	
8	80/F	6	

In all cases

(i) Body was shifted to mortuary freezer within 60 min after death

(ii) Mortuary freezer was maintained at 2–4 °C with temperature recording

(iii) At emergency services for the head injury victims, oxygen saturation was maintained by intubation and oxygen mask

(iv) All subjects received only anti-oedema measure, fluid correction and terminally one vial of adrenaline, atropine and steroid

into the deep freezer maintained at −80 °C. The postmortem time (PMI) (the elapsed time between death and the freezing of the brain samples following autopsy and dissection) was recorded for each case. Human brain areas chosen for the study were anatomically farthest from the site of injury and without distinct edema or abnormal morphology. While the major portion of the tissue was frozen for biochemical studies, a minor portion corresponding to the mirror image bits of the stored tissue were fixed in buffered formalin. These tissues were subjected to routine histological assessment (by SKS and AM) as indicated below and the samples that maintained tissue integrity were utilized for the study (data not shown). The same procedure of autopsy, tissue handling and other protocols were uniformly maintained for all the samples in this study. Similar PD and control tissues from the human brain bank have been earlier utilized extensively in PD research [18, 30–32].

In the current study, frontal cortex (FC) region was analyzed in the PD and non-PD control brains [18]. The nigrostriatum could not be examined due to the non-availability of tissue from the cases of PD brains, following earlier studies. Table 1 shows the details related to the brain samples including age, gender, PMI and neurological score. The age of the controls [Mean (SD): 61.5 ± 14.9 years, range: 35–77 years; n = 8; 6 males, 2 females)] and PD (62.4 ± 14.1 years, range = 35–80 years; n = 9; 8 males and 1 female) are indicated. The average postmortem delay was 13.8 ± 6.4 h (range = 6–22 h) in controls and 6.1 ± 5.1 h (range = 1.5–15 h) in PD.

Isolation of Neuropil Fractions from the Brain Tissue [33]

Approximately 200 mg of brain tissue (FC) was minced in 1,000 µl of isolation Buffer (350 mM sucrose, 5 mM TES and 1 mM EGTA, pH 7.2) containing 100 µl of protease inhibitor cocktail and manually homogenized (16 strokes). The homogenate was centrifuged at 1,000×*g* for 5 min at 4 °C and the supernatant was stored. The pellet was homogenized again (16 strokes) on ice and centrifuged (1,000×*g*, 5 min at 4 °C). The supernatants from both the steps were pooled and centrifuged at 8,500×*g* for 10 min at 4 °C to obtain the crude mitochondrial/synaptosomal pellet. The post-mitochondrial fraction which corresponded to the cytosolic fraction was aliquoted and stored. The pellet was re-suspended in 300 µl of isolation buffer and overlaid on a discontinuous Ficoll gradient consisting of 6 % (w/v Ficoll, ρ = 1.065 g/ml, 3 ml), 9 % (w/vFicoll, ρ = 1.075 g/ml, 2 ml) and 12 % (w/v Ficoll, ρ = 1.085 g/ml, 3 ml). The gradient was centrifuged at 75,000×*g* for 1 h at 4 °C and the synaptosomes which formed a whitish ring in the middle of the gradient was collected, resuspended in reconstitution buffer (250 mM sucrose and 10 mM TES, pH 7.2) and stored as aliquots in −80 °C. Similarly, the mitochondria which formed the pellet were carefully resuspended in the reconstitution buffer. We previously demonstrated that the mitochondria and synaptosomes isolated from human brain samples by this method maintained purity and integrity [34–36]. All the three fractions were utilized for biochemical assays after protein estimation [37].

SDS PAGE and Western Blot

Soluble protein (50 µg) from different samples was loaded on 10 % SDS PAGE followed by western blot with either anti-GFAP or β-actin antibody [18].

Estimation of Protein Carbonyls (Oxyblot) and Protein Nitration

Oxyblots were carried out as described earlier [22, 38]. Briefly, protein extract (4 mg/ml) was derivatized by

dinitrophenyl hydrazine (DNPH) in a 20 µl reaction mixture in the presence of 12 % SDS for 20 min at room temperature. The reaction was stopped by neutralization with 2 M Tris in 30 % glycerol and 5 µl of the sample was spotted in triplicate on nitrocellulose membrane and probed with anti-DNP antibody. Non-derivatized samples did not show anti-DNP immunoreactivity confirming the specificity of the antibody (data not shown).

To detect protein nitration, protein (10 µg/sample) from different samples were spotted in triplicate onto a nitrocellulose membrane and probed with polyclonal anti-3-nitrotyrosine (3-NT) antibody. Western blot signals were densitometrically quantified and normalized against the respective anti-β-actin signal [22].

Assays for Antioxidant Function and GSH Metabolism

Superoxide Dismutase (SOD) Assay

SOD activity was assayed using its inhibitory action on quercetin oxidation based on the method described earlier with minor modifications [39]. The final reaction mixture contained 30 mM Tris HCl (pH 9.1), 0.5 mM EDTA, 50 mM TEMED, 0.05 mM quercetin and 10 µl of brain extract supernatant containing 10 µg of protein. The reaction was monitored at 406 nm for 10 min. One unit of SOD activity was defined as the amount of enzyme (per mg protein) that inhibits quercetin oxidation reaction by 50 % of the maximal value.

Estimation of Total Glutathione (GSH + GSSG)

In all the samples, total glutathione (GSH + GSSG) and GSSG content were estimated. Total glutathione estimations were carried out by the 5,5′ dithio-bis-2-nitro benzoic acid recycling method as described earlier [40]. All estimations were conducted in triplicate and total glutathione concentrations were normalized per mg protein.

GSSG content in the neuropil fractions was measured by the method described previously [41] with minor modifications. Soluble extract prepared as described previously [40] was incubated first with 0.05 M N-ethylmaleimide (NEM) for 20 min to block the reduced GSH in the sample. Later, the reaction mixture containing 10 µl of sample, 180 µl of 0.1 N NaOH, and 10 µl o-phthalaldehyde (20 mg/ml) was incubated at room temperature for 15 min and the fluorescence (excitation at 350 nm and emission at 420 nm) was recorded. All the values were obtained in triplicate, compared with GSSG standards and normalized per mg protein.

Glutathione Peroxidase (GPx) Assay

GPx activity was determined by t-butyl hydroperoxide (tbHP) method [42]. The reaction mixture containing 150 µg protein (sample), 0.1 M phosphate buffer, 0.5 mM EDTA, 100 µl Glutathione reductase (0.24 U), 100 µl GSH (1 mM), and 100 µl NADPH (0.15 mM) was incubated at 37 °C for 3 min and the reaction was initiated by the addition of 100 µl tbHP (0.12 mM). Change in absorbance at 340 nm was monitored for 5 min spectrophotometrically and the activity was expressed as ηmoles of NADPH oxidized/min/mg protein (MEC = 6.22 $mM^{-1}cm^{-1}$).

Glutathione Reductase (GR)

Solubilized brain protein extract (100 µg) was assayed at 25 °C in 0.1 M Tris–HCl (pH 8.1) and 0.2 mM NADPH and the reaction was initiated by the addition of 1 mM GSSG. The enzyme activity was measured by monitoring the oxidation of NADPH, spectrophotometricallyat 340 nm [43].

Glutathione-S-Transferase (GST) Assay

GST was assayed by the 1-chloro 2-4-dinitro benzene (CDNB) method [44]. To 1 ml reaction mixture containing phosphate buffer (0.1 M, pH 6.5), 0.5 mM EDTA, CDNB (1.5 mM) and 50 µl GSH (1 mM), 30 µg protein (sample) was added and the increase in absorbance at 340 nm was monitored for 5 min. The enzyme activity was expressed as nmoles of S-2,4, dinitrophenyl glutathione formed/min/mg protein (MEC = 9.6 mM^{-1} cm^{-1}).

Assays for Mitochondrial Function

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT assay, which is a colorimetric method that measures the reduction of MTT by mitochondria reductases was carried out as described previously [35, 45]. Briefly, 5 µl of the sample (~5 µg protein) was added to 1,000 µl of buffer (Mannitol-Sucrose-HEPES, 20 mM sodium succinate, 1 mM NADH, pH 7.4). To this, 15 µl of MTT (5 mg/ml) was added and incubated at 37 °C for 2 h. The formazan crystals formed, were dissolved in 100 µl SDS-dimethyl formamide (DMF) buffer (45 % DMF in distilled water and 10 % SDS, pH 4.7), and the absorbance was measured at 570 nm.

Citrate Synthase (CS)

CS activity which represents the rate of reduction of 5, 5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to

thionitrobenzoic acid (TNB) at 412 nm was assayed as described previously [35, 46]. The reaction was initiated by the addition of 20 µl of 10 mM oxaloacetate (final concentration 0.2 mM) to the reaction mixture containing 100 mM Tris–HCl (pH 8.1), 0.2 mM DTNB, 0.1 % Triton X-100, 0.1 mM acetyl-CoA and 20 µg of mitochondrial protein. The results were expressed as nmol DTNB/min/mg protein (MEC = 13.6 $mM^{-1}cm^{-1}$).

Succinate Dehydrogenase (SDH)

SDH activity was determined as an end-point assay based on the method of Pennington [47] with minor modifications [35]. Mitochondrial sample (10 µl) was added to 70 µl of 0.01 mol/l sodium succinate solution in 0.05 mol/l phosphate buffer, pH 7.5 and incubated for 15 min at 37 °C. Next, 70 µl of 1 µg/µl p-iodonitrotetrazolium violet was added to the reaction and incubated at 37 °C for an additional 10 min. The reaction was stopped by the addition of 100 µl of a 5:5:1 (v:v:w) solution of ethyl acetate:ethanol:trichloroacetic acid and the absorbance was measured at 490 nm. The specific activity was expressed as OD/mg protein.

Malate Dehydrogenase (MDH)

Malate dehydrogenase activity was assayed as described previously [35, 48]. To 230 µl of the reaction buffer [Potassium phosphate buffer (0.1 M, pH 7.5), 0.03 ml NADH (14.3 mM) and 0.05 ml oxaloacetate (20 mM)], mitochondrial sample (10 µg) was added and the decrease in absorbance due to increased oxidation of NADH was measured for 3 min at 340 nm. The enzyme activity was expressed as nmoles NADH/min/mg protein (MEC = 6.22×10^{-6} $nmol^{-1}$ cm^{-1}).

Statistical Analysis

Quantitative data from at least three independent experiments were expressed as mean ± SD followed by analysis of variance (ANOVA). For all the quantitative data, $p < 0.05$ was considered to be statistically significant.

Results

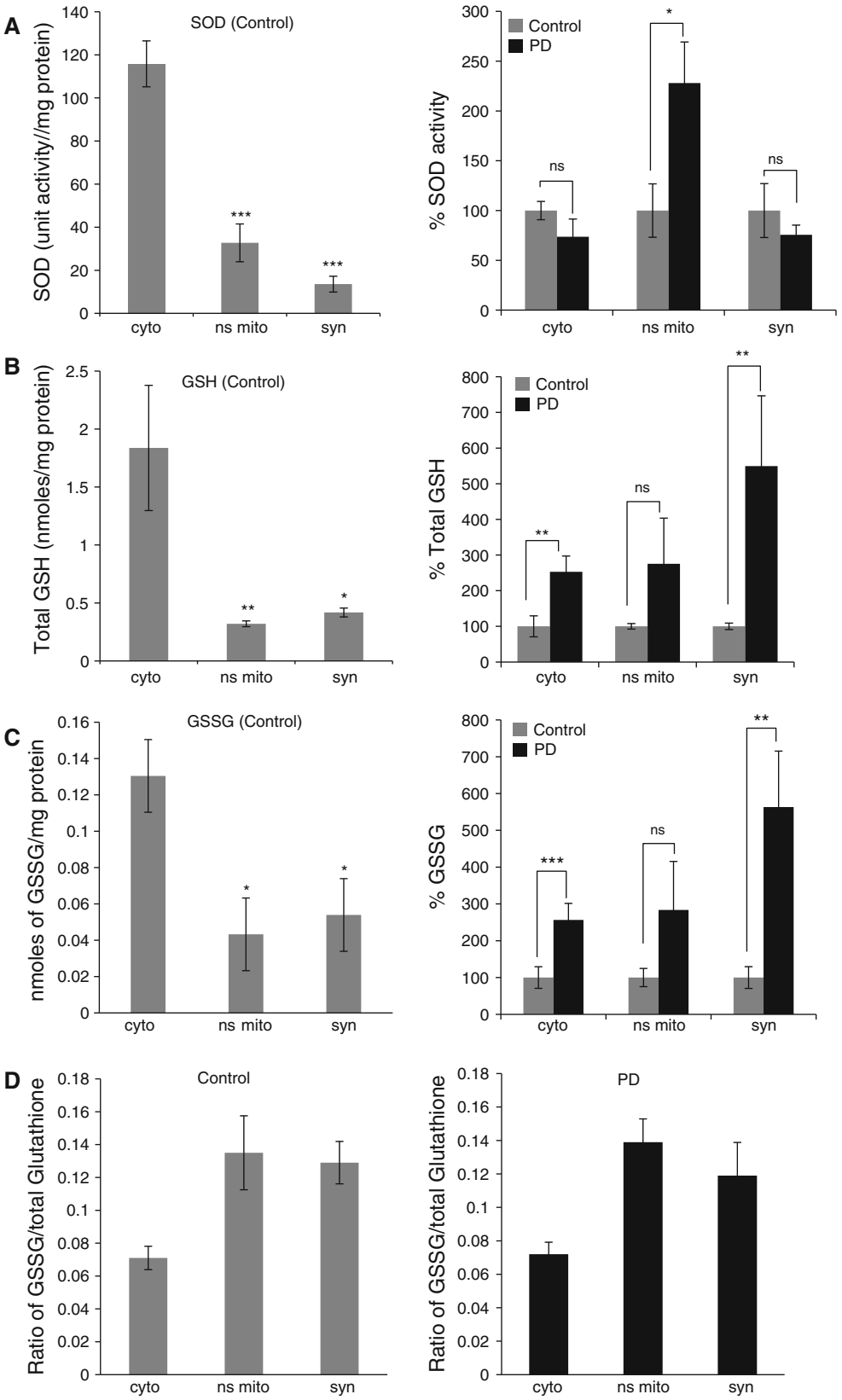
Experimental evidences in PD have demonstrated significant oxidative stress and mitochondrial dysfunction in the SN [49–51]. In our previous study [18], we observed that compared to SN, non-SN regions such as FC were protected from oxidative and mitochondrial damage due to increased GSH. In the current study, we investigated the distribution of antioxidant markers in different sub-cellular fractions in FC in the brains from cases of PD compared to controls. This is physiologically relevant since the activity in total brain extract might not reflect the status of different markers in the neuropil compartments and different fractions might respond differently to oxidative stimuli. We observed this phenomenon of differential response both in control and PD brains.

Fig. 1 Alterations in the antioxidant activities in the neuropil fractions of FC region of human PD brains (n = 9) and controls (n = 6). Neuropil fractions (cytosol-cyto, non-synaptic mitochondria-ns mito, synaptosomes-syn) were isolated from FC. SOD activity and total GSH content in all the fractions were assayed in triplicate and the average for each sample was plotted as mean ± SD followed by ANOVA analysis and calculation of *p* values. The *bar graph* and the *p* values in the neuropil fractions of control and PD samples: **a** SOD activity (Absolute SOD activity in cytosol = 115.8 unit activity/mg protein, mitochondria = 32.7 unit activity/mg protein and synaptosome = 13.6 unit activity/mg protein. SOD activity in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples) **b** Total GSH (Total GSH content in cytosol = 1.8 nmoles/mg protein, mitochondria = 0.32 nmoles/mg protein and synaptosome = 0.4 nmoles/mg protein. The total GSH in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples). **c** Total GSSG (Total GSSG content in cytosol = 0.13 nmoles/mg protein, mitochondria = 0.04 nmoles/mg protein and synaptosome = 0.05 nmoles/mg protein. The total GSSG in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples) and **d** GSSG/total glutathione ratio from the control and PD samples. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, not significant (ns) compared to the respective controls or compared to cyto

In our earlier study, we had reported that the total SOD activity in non-SN regions of the brain was unaltered in PD compared to control [18]. However, the current study showed that the SOD activity in the FC from control brains were ~4 fold lower in the mitochondria ($p < 0.001$) and ~10 fold lower in the synaptosomes ($p < 0.001$) compared to the cytosolic fraction (Fig. 1a). Further, while the SOD activity in the cytosol and synaptosomes of FC tissue from PD cases was unaltered, mitochondrial SOD activity was found to be increased by ~2 fold ($p = 0.017$) compared to control (Fig. 1a).

In the control brains, total GSH displayed ~5 fold lower level in mitochondria ($p < 0.01$) and synaptosomes ($p < 0.05$) compared to cytosolic fraction (Fig. 1b). On the other hand, we observed distinct elevation in GSH content in the synaptic terminals of the FC from PD brains. Accordingly, synaptosomes from PD brains showed ~5 fold elevation in total GSH compared to control ($p = 0.01$), while the cytosolic GSH was elevated by ~2.5 fold compared to controls ($p = 0.009$). Interestingly, the mitochondrial GSH content was unaltered in PD samples ($p = 0.23$) (Fig. 1b).

To investigate whether the elevation in total glutathione was associated with the conversion of GSH to GSSG, we estimated the GSSG in the neuropil fractions. The GSSG


A
SOD (Control)
SOD (unit activity//mg protein)
140
120
100
80
60
40
20
0
***
***
cyto
ns mito
syn
% SOD activity
Control
PD
300
250
200
150
100
50
0
*
ns
ns
cyto
ns mito
syn
B
GSH (Control)
Total GSH (nmoles/mg protein)
2.5
2
1.5
1
0.5
0
**
*
cyto
ns mito
syn
% Total GSH
Control
PD
800
700
600
500
400
300
200
100
0
**
ns
**
cyto
ns mito
syn
C
GSSG (Control)
nmoles of GSSG/mg protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
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cyto
ns mito
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Control
PD
800
700
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200
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cyto
ns mito
syn
D
Control
Ratio of GSSG/total Glutathione
0.18
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0.14
0.12
0.1
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0.04
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Ratio of GSSG/total Glutathione
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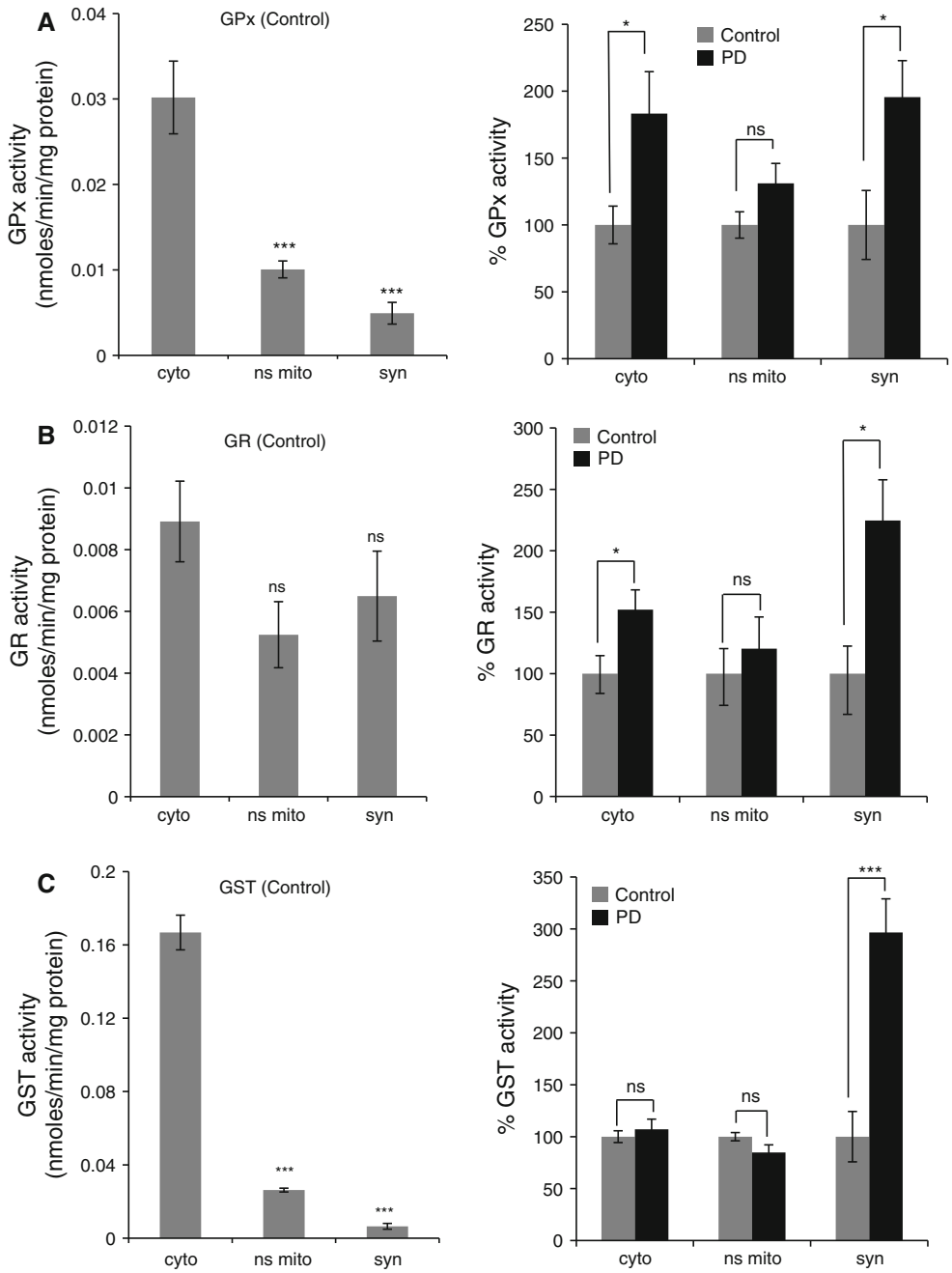

Fig. 2 Alterations in the activities of GSH enzymes in the neuropil fractions of FC region of human PD brains (n = 9) and controls (n = 6). Neuropil fractions (cytosol-cyto, non-synaptic mitochondria-ns mito, synaptosomes-syn) were isolated from FC. GPx, GR and GST activities in all the fractions were assayed in triplicate and the average for each sample was plotted as mean ± SD followed by ANOVA analysis and calculation of *p* values. The *bar graph* and the *p* values in the neuropil fractions from control and PD samples are shown as follows: **a** GPx activity (Total GPx activity in cytosol = 0.03 nmoles/min/mg protein, mitochondria = 0.01 nmoles/min/mg and synaptosome = 0.005 nmoles/min/mg. The GPx activity in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples). **b** GR activity (Absolute GR activity in cytosol = 0.009 nmoles/min/mg protein, mitochondria = 0.005 nmoles/min/mg and synaptosome = 0.0065 nmoles/min/mg. GR activity in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples) **c** Total GST activity (Absolute GST activity content in cytosol = 0.17 nmoles/min/mg, mitochondria = 0.03 nmoles/min/mg and synaptosome = 0.006 nmoles/min/mg. The GST activity in the respective neuropil fraction of control brains was considered as 100 % for comparison with PD samples) $*p < 0.05$, $***p < 0.001$, not significant (ns) compared to the respective controls or compared to cyto

Table 2 Comparison of antioxidant activities in the sub-cellular compartments (neuropil fractions) of FC tissue of postmortem human brains from control subjects (n = 8) and PD cases (n = 9)

Sl. no.	Assay	Control samples: high/low compared to cytosol (with p value)	PD samples: high/low compared to control (with p value)
1.	SOD	Mitochondria = low ($p < 0.001$)	Mitochondria = high ($p < 0.05$)
		Synaptosomes = low ($p < 0.001$)	Synaptosomes = ns
			Cytosol = ns
2.	Total GSH	Mitochondria = low ($p < 0.01$)	Mitochondria = ns
		Synaptosomes = low ($p < 0.05$)	Synaptosomes = high ($p < 0.01$)
			Cytosol = high ($p < 0.01$)
3.	GPx	Mitochondria = low ($p < 0.001$)	Mitochondria = ns
		Synaptosomes = low ($p < 0.001$)	Synaptosomes = high ($p < 0.05$)
			Cytosol = high ($p < 0.05$)
4.	GR	Mitochondria = ns	Mitochondria = ns
		Synaptosomes = ns	Synaptosomes = high ($p < 0.05$)
			Cytosol = high ($p < 0.05$)
5.	GST	Mitochondria = low ($p < 0.001$)	Mitochondria = ns
		Synaptosomes = low ($p < 0.001$)	Synaptosomes = high ($p < 0.001$)
			Cytosol = ns

Neuropil fractions [cytosol, non-synaptic mitochondria (mitochondria) and synaptosomes] were isolated from control and PD brains followed by analysis of antioxidant activities. The data from the mitochondrial and synaptosomal fractions for different activities compared to the corresponding cytosolic value (high/low) and it significance (p value) are shown. Similar data from PD FC samples compared to the age-matched control are shown

SOD Superoxide dismutase, *GSH* glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *GST* glutathione-s-transferase, *ns* not significant

content in the control samples showed a trend similar to the total glutathione (Fig. 1b, c), with the cytosolic GSSG significantly higher that the mitochondrial and synaptosomal GSSG ($p < 0.05$). In the PD samples, the synaptosomes showed the highest percentage increase in GSSG ($p < 0.01$) compared to control followed by cytosol ($p < 0.001$), while it was unchanged in the non-synaptic mitochondria. The ratio of GSSG to total glutathione (GSH + GSSG) in the different fractions did not show any difference between the control and PD samples indicating that the elevated total glutathione was not due to conversion of GSH to GSSG (Fig. 1d).

The GPx activity in the control brains was ~3 fold lower in mitochondria ($p < 0.001$) and ~5 fold lower in synaptosomes ($p < 0.001$) compared to cytosol (Fig. 1c, inset). On the other hand, GPx activity in the PD tissue was elevated by ~2 fold both in the cytosol ($p = 0.02$) and synaptosomes ($p = 0.02$) compared to control, while it was unaltered in mitochondria ($p = 0.09$) (Fig. 2a).

GSH reductase activity in the control FC showed similar activity in all neuropil fractions (Fig. 2b). However, the GR activity in the FC from PD brains was elevated by ~2.5 fold in synaptosomes ($p = 0.006$) and by ~1.5 fold in the cytosol ($p = 0.03$) compared to the respective controls, while it was unaltered in mitochondria ($p = 0.54$) (Fig. 2a). On the other hand, GST in the control brains showed ~6 fold lower activity ($p < 0.001$) and >10 fold lower activity in synaptosomes ($p < 0.001$) compared to cytosol. GST activity in the FC from PD brains was significantly elevated on in the synaptosomes by ~3 fold ($p = 0.0001$) while it was unaltered in cytosolic and mitochondrial fractions (Fig. 2c).

These data indicated that (1) the antioxidant markers showed significant alteration in control brains across different sub-cellular fractions of FC with the synaptosomes showing least activity followed by mitochondria and cytosol (2) synaptosomes from FC of PD brains showed relatively increased GSH content and higher related enzyme activities compared to controls (3) mitochondria from FC of PD brains showed higher SOD activity compared to controls (Table 2).

Next, we investigated whether markers of oxidative stress in different fractions showed alteration in PD compared to age-matched controls. The mitochondrial fraction showed lowered protein nitration levels (indicated by 3-nityrotyrosine or 3-NT blot) ($p < 0.01$) and lowered protein oxidation (indicated by anti-dinitrophenyl or anti-DNP blot) in PD samples compared to controls ($p < 0.05$) (Fig. 3). Similarly, protein nitration was unaltered ($p = 0.5$) and protein oxidation was lowered in PD synaptosomes ($p < 0.01$) compared to controls (Fig. 4). The cytosolic fraction from the FC of PD brains showed lowered protein oxidation ($p < 0.05$), while the protein 3-NT level ($p = 0.13$) and GFAP expression ($p > 0.05$) were unaltered (Fig. 5). These observations indicate protection against oxidative damage

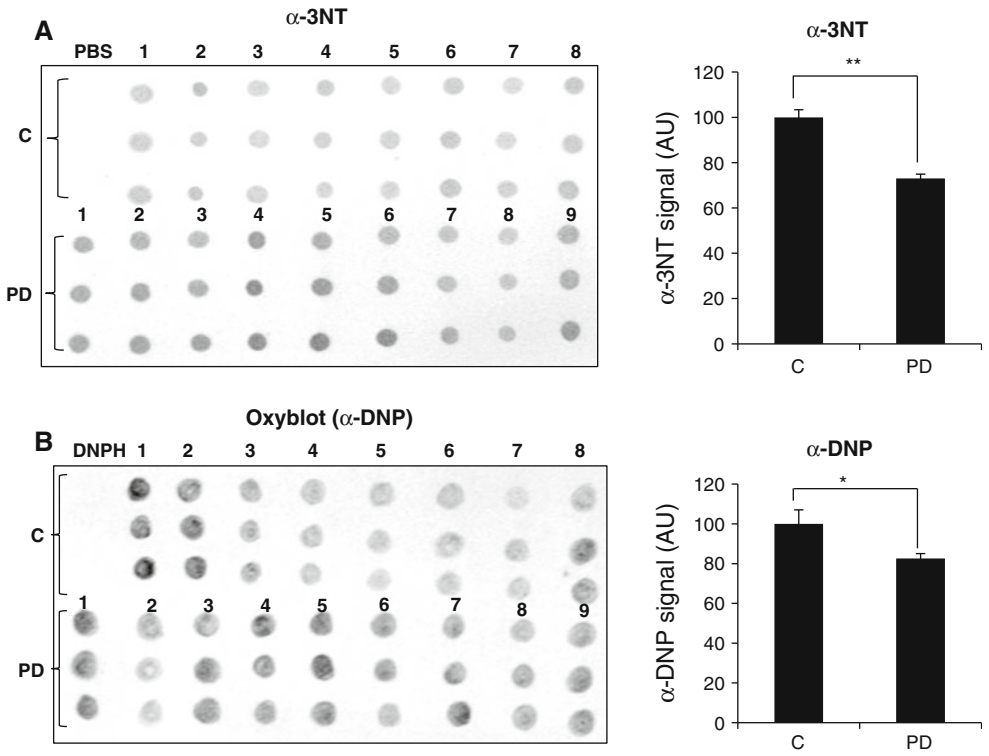

Fig. 3 Analysis of markers of oxidative and nitrosative stress in non-synaptic mitochondria (ns mito) FC from human PD brains (n = 9) and controls (n = 6). Total protein extracts (100 μg) from FC (ns mito) of Control (C) and PD samples were spotted on nitrocellulose membrane followed by anti-3NT western blot. **a** representative anti-3NT blot from C and PD (in triplicate indicated by flower bracket) and its quantitation based on densitometric analysis are indicated in the figure. PBS corresponds to no-protein control. Total protein extract after DNP-derivatization (~10 μg) in FC from C and PD samples were spotted on nitrocellulose membrane in triplicate followed by anti-DNP western blot (Oxyblot). **b** Representative anti-DNP blot from C and PD (in triplicate indicated by flower bracket) and its quantitation based on densitometric analysis are shown. DNPH corresponds to the sample with DNPH only and without protein. $*p < 0.05$, $**p < 0.01$, *ns* not significant

by elevated SOD in the mitochondria and elevated GSH content in the synaptosomes and cytosolic compartments.

Next, we investigated whether the difference in the antioxidant status between synaptosomes and non-synaptic mitochondria is associated with the quantum of mitochondrial content and function. To address this issue, we carried out mitochondrial assays including MTT, CS, SDH and MDH in both fractions (Fig. 6). Based on these functional assays, we observed that the synaptosomes showed ≥5 fold lower mitochondrial content compared to the non-synaptic mitochondria, both in the control and PD samples.

Discussion

Even as previous studies have correlated the redox dynamics [4, 7–14] and mitochondrial status [15, 16] in PD brains compared to controls, the sub-cellular distribution of antioxidant markers in PD brains need to be investigated, as such studies in human samples are non-existent. The current study compared non-synaptic mitochondria, synaptosomes (which represent synaptic terminals) and cytosolic fractions from human PD brains. While the total extracts from the human brain represent neuronal and non-neuronal cells, analysis of synaptosomes represents only the neuronal physiology devoid of glial and vascular endothelial contribution. Since the bioenergetic capacities of neurochemically different synaptosomes do not vary significantly [52], the synaptosomes used in the current study could be considered as a homogenous population. We observed that the synaptosomes and mitochondrial fractions in the control FC showed several fold lower antioxidant activity compared to cytosol (Figs. 1, 2).This is in agreement with our previous study [36], which showed that the antioxidant activities such as SOD, GPx and GST in the neuronal cytosol and synaptosomes were altered by age. Ansari et al. [53] showed that the neuropil fractions from subjects with mild cognitive impairment and Alzheimer's disease (AD) displayed elevated oxidative stress and antioxidant depletion, with the highest oxidative damage observed in the synaptosomes. While these data indicate the selective vulnerability of synaptosomes, elevated GSH and related activities in FC of PD brains (Figs. 1, 2) might be neuroprotective, consistent

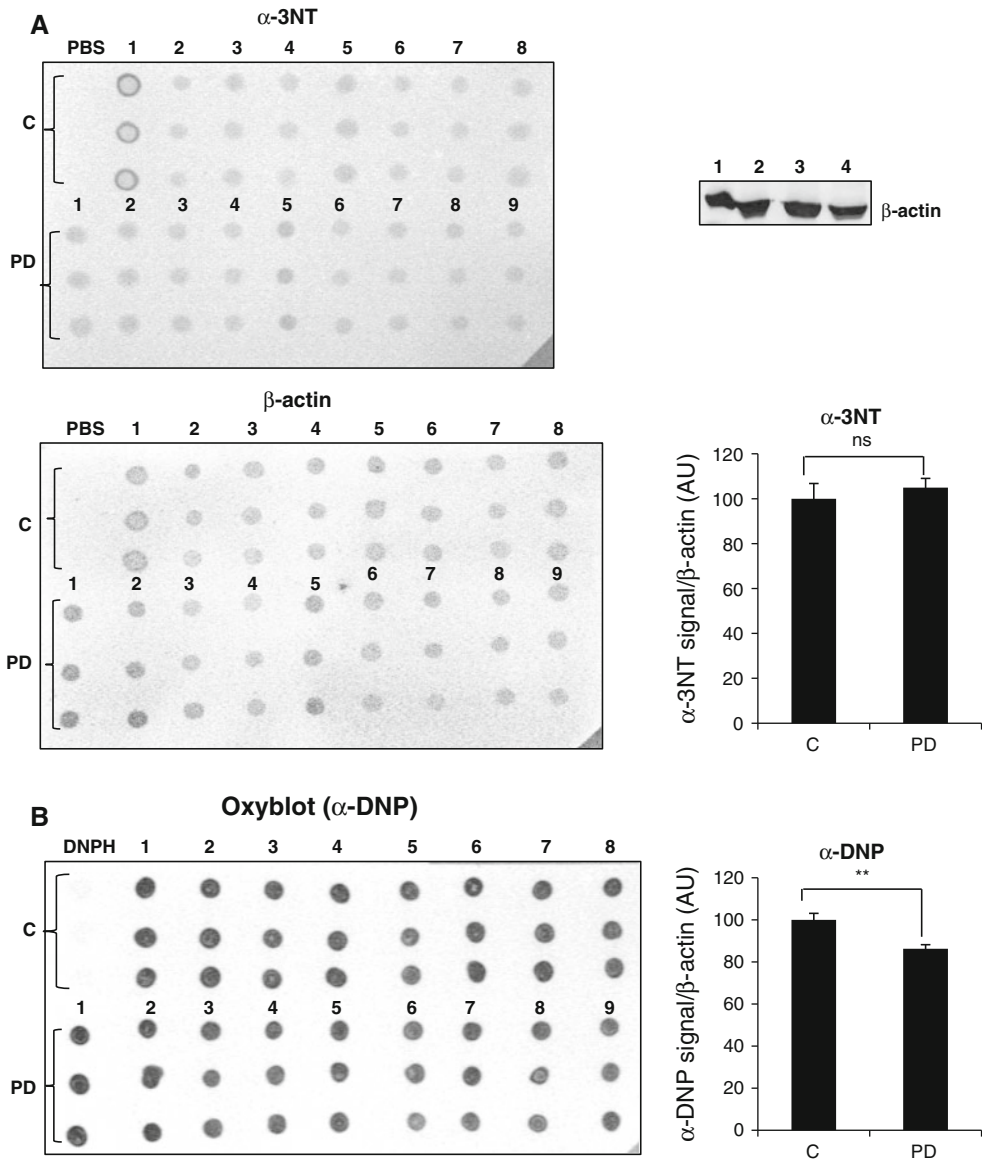

Fig. 4 Analysis of markers of oxidative and nitrosative stress in the synaptosomal fraction (syn) of FC from human PD brains (n = 9) and controls (n = 6). Total protein extracts (100 μg) from FC (synaptosmes or syn) of Control (C) and PD samples were spotted on nitrocellulose membrane followed by anti-3NT western blot. **a** Representative anti-3NT blot and representative western blot (from 4 samples) and dot blot for *β*-actin from C and PD samples (in triplicate indicated by flower bracket) followed by quantitation of anti-3NT signal normalized with *β*-actin signalare shown. PBS corresponds to no-protein control. Total protein extract after DNP-derivatization (~10 μg) in FC syn from C and PD samples were spotted on nitrocellulose membrane in triplicate followed by anti-DNP western blot (Oxyblot). **b** Representative anti-DNP blot of FC from PD and C samples and quantitation of anti-DNP signal normalized with *β*-actin signal are shown. DNPH corresponds to the sample with DNPH only and without protein as indicated in the figure. $^{**}p < 0.01$, ns = not significant

with a report [54] that elevated brain GSH protected synaptosomes against acrolein-induced damage. While higher GSH content and related enzyme activities might provide the major antioxidant defense in the synaptosomes, elevated SOD activity might play an important role in the non-synaptic mitochondria. Interestingly, most of the other activities were relatively unaltered in the non-synaptic mitochondria (Figs. 1, 2). Two previous studies [55, 56] showed that under conditions that induce cellular GSH depletion, irrespective of the in vivo GSH levels, the mitochondrial GSH pool was unaltered thus signifying the mitochondrial mechanism underlying the ability to counter oxidative stress.

These results also indicate that the data from whole cell or tissue extracts might not reflect the status of different activities in individual compartments. Hence biochemical

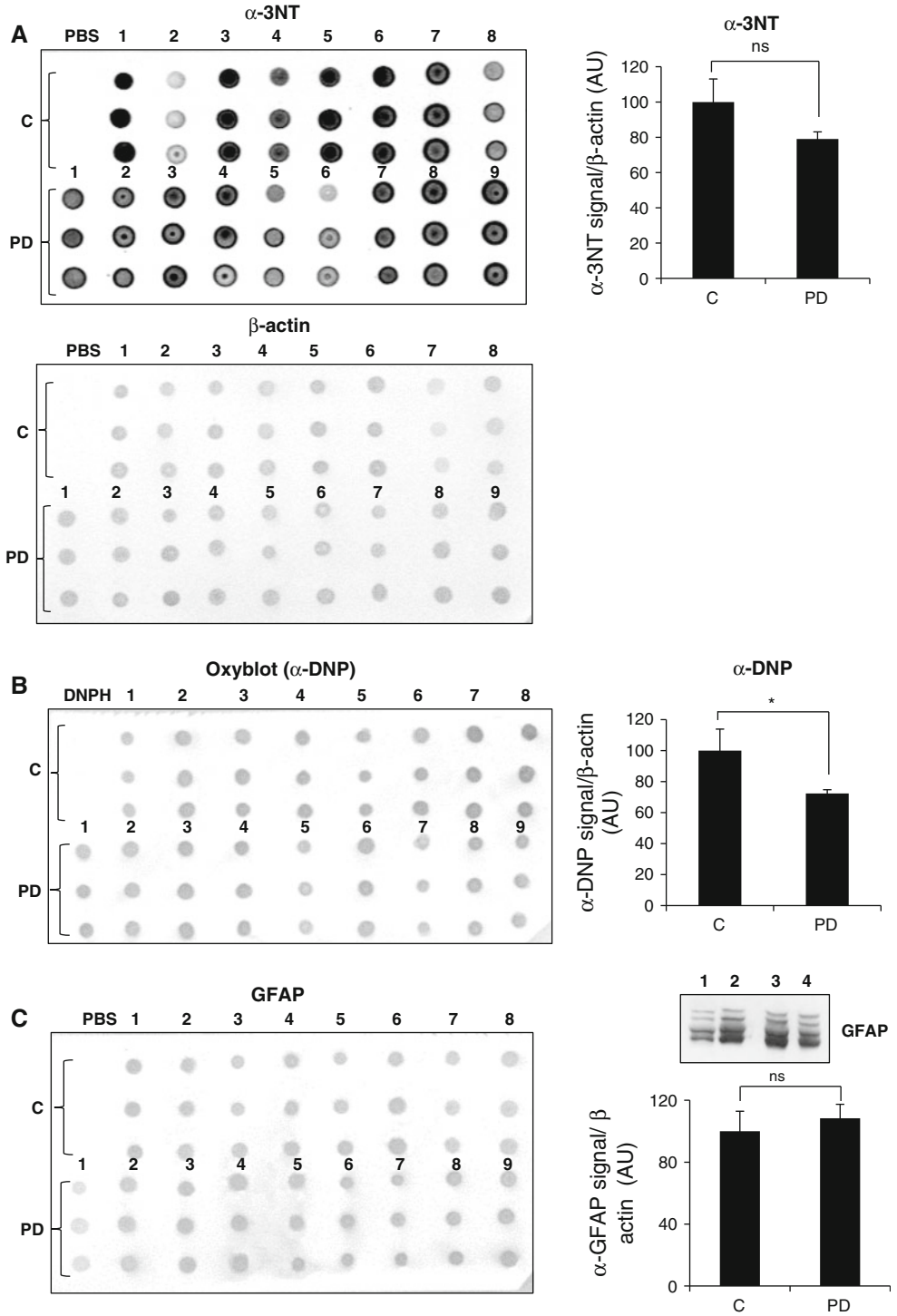

Fig. 5 Analysis of markers of oxidative and nitrosative stress and GFAP expression in the cytosolic fraction of FC from human PD brains (n = 9) and controls (n = 6). Total protein extracts (100 μg) from FC (cytosol) of Control (C) and PD samples were spotted on nitrocellulose membrane followed by anti-3NT western blot. **a** representative anti-3NT dot blot and *β*-actin dot blot from C and PD (in triplicate as indicated by flower bracket) and quantitation of anti-3NT signal normalized with *β*-actin signal are shown. PBS corresponds to no-protein control. Total protein extract after DNP-derivatization (∼10 μg) in FC (PMS) from C and PD samples were spotted on nitrocellulose membrane in triplicate followed by anti-DNP western blot (Oxyblot). **b** Representative anti-DNP blot of FC tissue (cytosol) showing PD and C samples and quantitation of anti-DNP signal normalized with *β*-actin signal are shown. **c** Representative anti-GFAP western blot (from four samples) and dot blot of FC tissue (cytosol) showing PD and C samples and quantitation of anti-GFAP signal normalized with *β*-actin signal are shown. PBS corresponds to no-protein control. $*p < 0.05$, *ns* not significant

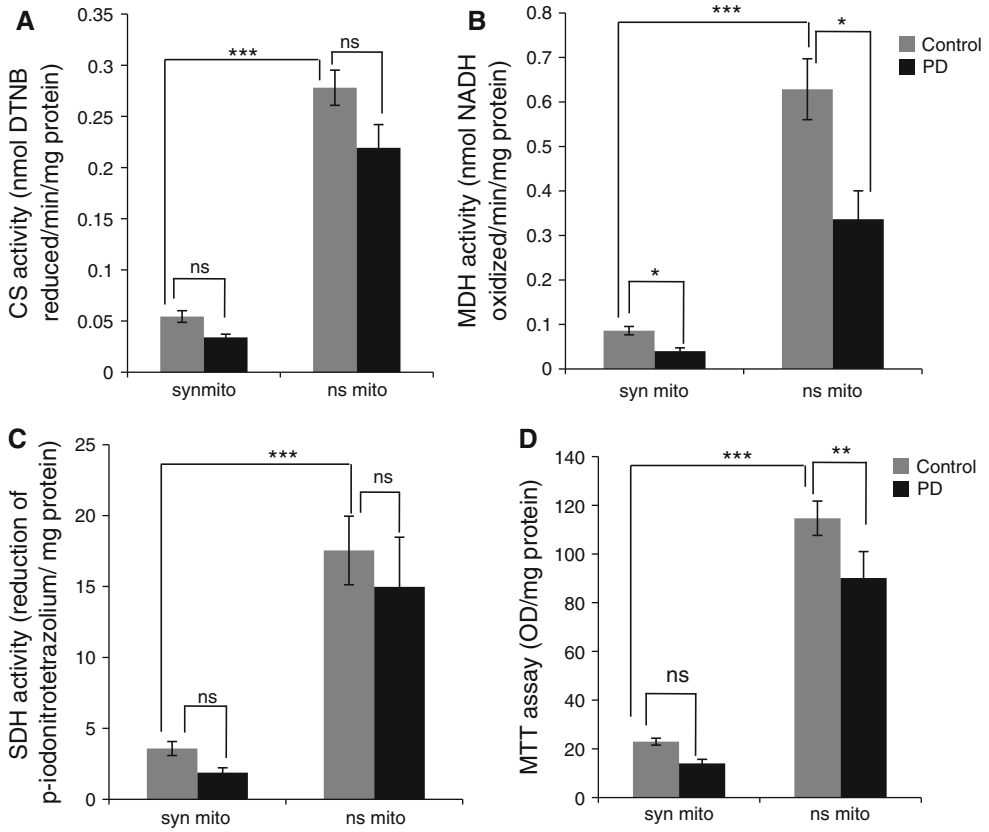

Fig. 6 Analysis of mitochondrial enzyme activities in the synaptic and non-synaptic mitochondria of FC region of human PD brains (n = 9) and controls (n = 6). Non-synaptic mitochondria (ns mito), synaptic mitochondria (syn mito) were isolated from FC. CS, MDH, SDH and mitochondrial reductase (MTT) activities in both the fractions were assayed in triplicate and the average for each sample was plotted as mean ± SD followed by ANOVA analysis and calculation of *p* values. The *bar graph* and the *p* values in the neuropil fractions from control and PD samples are shown as follows: **a** CS activity (expressed as nmoles of DTNB reduced/min/mg protein) **b** MDH activity (expressed as nmoles of NADH oxidized/min/mg protein) **c** SDH activity (expressed as OD/mg protein (OD as a result of reduction of p-iodonitrotetrazolium/mg protein) and **d** MTT values (OD as a result of reduction of MTT to formazan/mg protein). $*p < 0.05$, $***p < 0.001$, not significant (ns) compared to the respective controls

activity from total cell extracts should be interpreted cautiously. In a relevant study on aging human brains [36], we showed that while the SOD activity and GSH content were unchanged with increasing age in total brain extracts, the parameters changed significantly in the neuropil fractions. In another study [18] carried out on the total extract of FC from PD brains, we showed that the SOD activity from PD brains was unaltered compared to controls, while the SOD activity was elevated in the non-synaptic mitochondria (Fig. 1a), consistent with a previous study [57] which demonstrated increased mitochondrial SOD activity in the motor cortex from PD cases but not in cases of amyotropic lateral sclerosis. Similarly, while the GR and GST activities in the total extract of FC were unaltered between PD and control brains[18] neuropil analysis revealed significantly elevated activities in the synaptosomes (Fig. 2b, c). Total GSH was elevated by 3 fold in total extract of FC compared to control [18] while the highest relative elevation was observed in the synaptosomes (Fig. 1b) compared to cytosol and non-synaptic mitochondria. On the other hand, the GPx activity elevated in the total extract of FC from PD brains was contributed only by the cytosolic and synaptosomal fractions while the activity was unchanged in the mitochondria (Fig. 2a).

In the human brains obtained after autopsy, pre-mortem (age, gender, agonal state) and postmortem factors [post-mortem interval (PMI), storage time] might impinge on tissue integrity and quality [35, 58]. PMI might affect different biochemical parameters in the human brain tissue [22].The average PMI of the control brain samples (13.8 h) used in the current study was nearly two-fold higher than in the PD samples (6.1 h), which might affect the antioxidant activities. However, our previous studies on human brains showed that increasing PMI (2.5–26 h) did not affect the antioxidant activities, GSH content and related enzymes in FC [59]. In another study, we have demonstrated that increasing PMI did not influence the postmortem stability of glial fibrillary acidic protein (GFAP) and oxidatively damaged proteins indifferent anatomical regions of human

brains including FC [58]. These studies indicate that the oxidant and antioxidant markers in FC are relatively unaffected by PMI and the differences observed between the control and PD samples in the study represent disease-related changes.

Most studies on redox dynamics in PD have concentrated on the nigrosriatum but studies on FC are limited [60].While the SN from PD brains displays GSH loss [61, 62], non-SN regions show elevated GSH content, which might protect mitochondria against oxidative damage [18]. Elevated GSH could either be due to increased synthesis or leakage of intracellular GSH or by lowered breakdown. We previously showed that the elevated GSH in FC of PD brains was due to lowered activity of gamma glutamyl transpeptidase (GGT), while the activity of the GSH synthesizing enzyme gamma glutamyl ligase (GCL) was unaltered [18]. The current study showed that the GSH elevation in FC was not due to the oxidation of GSH to GSSG since both control and PD samples showed the same GSSG/total glutathione ratio (Fig. 1c, d). Analysis of mitochondrial enzyme activities (Fig. 6) indicated that the mitochondrial content in the synaptosomal fraction was at least 5 fold lower than the non-synaptic mitochondria. Since this phenomenon was seen both in the control and PD samples, it could be concluded that the selective vulnerability of the synaptosomes to oxidative damage in the controls and the relative changes in the antioxidant activities in PD samples is not directly associated with the mitochondrial content.

In conclusion, our data indicate that (1) the relative distribution of antioxidant markers including GSH in synaptic and non-synaptic regions significantly impinges on the degenerative and protective mechanisms in PD (2) synaptosomal and mitochondrial fractions exhibit lower antioxidant activity compared to cytosol in FC from control brains indicating the susceptibility of synaptosomes against oxidative damage (3) Elevated GSH content and related enzyme activities in FC of PD brains compared to controls might contribute to neuroprotection in PD.

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Conflict of interest The authors declare that there are no conflicts of interest.

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