

# Altered Expression of Brain Proteinase-Activated Receptor-2, Trypsin-2 and Serpin Proteinase Inhibitors in Parkinson's Disease

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**Abstract** Neuroinflammation is thought to contribute to cell death in neurodegenerative disorders, but the factors involved in the inflammatory process are not completely understood. Proteinase-activated receptor-2 (PAR2) expression in brain is increased in Alzheimer's disease and multiple sclerosis, but the status of PAR2 in Parkinson's disease is unknown. This study examined expression of PAR2 and endogenous proteinase activators (trypsin-2, mast cell tryptase) and proteinase inhibitors (serpin-A5, serpin-A13) in areas vulnerable and resistant to neurodegeneration in Parkinson's disease at different Braak  $\alpha$ -synuclein stages of the disease in post-mortem brain. In normal aged brain, expression of PAR-2, trypsin-2, and serpin-A5 and serpin-A13 was found in neurons and microglia, and alterations in the amount of immunoreactivity for these proteins were found in some brain regions. Namely, there was a decrease in neurons positive for serpin-A5 in the dorsal motor nucleus, and serpin-A13 expression was reduced in the locus coeruleus and primary motor cortex, while expression of PAR2, trypsin-2 and both serpins was reduced in neurons within the substantia nigra. There was an increased number

of microglia that expressed serpin-A5 in the dorsal motor nucleus of vagus and elevated numbers of microglia that expressed serpin-A13 in the substantia nigra of late Parkinson's disease cases. The number of microglia that expressed trypsin-2 increased in primary motor cortex of incidental Lewy body disease cases. Analysis of Parkinson's disease cases alone indicated that serpin-A5 and serpin-A13, and trypsin-2 expression in midbrain and cerebral cortex was different in cases with a high incidence of L-DOPA-induced dyskinesia and psychosis compared to those with low levels of these treatment-induced side effects. This study showed that there was altered expression in brain of PAR2 and some proteins that can control its function in Parkinson's disease. Given the role of PAR2 in neuroinflammation, drugs that mitigate these changes may be neuroprotective when administered to patients with Parkinson's disease.

**Keywords** Dyskinesia · L-DOPA · Neuroinflammation · Parkinson's disease · Proteinase-activated receptor-2 · Serpin (serine proteinase inhibitor clade A)

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## Introduction

Parkinson's disease is the most prevalent neurodegenerative movement disorder affecting 1 % of people over 65 years of age rising to 4 % of the population by the age of 80 years (Kowal et al. 2013). Pathologically, the disease is characterised by degeneration of brainstem nuclei and the accumulation of  $\alpha$ -synuclein-containing proteinaceous aggregates (Lewy bodies and neurites) throughout the brain. Dopaminergic neurons in the substantia nigra pars compacta are the most affected, and the resulting reduced dopamine levels in the caudate nucleus and putamen are responsible for the main motor symptoms of the disease that include bradykinesia,

rigidity, akinesia, abnormal posture and resting tremor. Non-motor symptoms also frequently occur (autonomic dysfunction, cognitive decline, depression), and these can be as incapacitating to the patient as the motor symptoms. Current pharmacological treatments restore dopamine levels (L-DOPA) or directly stimulate dopamine receptors (dopamine receptor agonists) in the caudate nucleus and putamen. Such treatments, although effective in restoring motor function, have little effect on non-motor symptoms, do not slow progression of the disease and have debilitating side effects (dyskinesia, psychosis) that limit their effective use (Lees et al. 2009).

Neuroinflammation occurs in brain regions that degenerate in Parkinson's disease and is thought to contribute to the neurodegenerative process (Barnum and Tansey 2012; McGeer et al. 1988). The hallmark of neuroinflammation in brain is the presence of activated microglia. These cells are resident in brain and are part of the innate immune system where they survey the local environment and clear cellular and/or bacterial debris by phagocytosis: activities that are both dependent on proteinases and their target receptors (Kettenmann et al. 2011). Non-steroidal anti-inflammatory drugs are neuroprotective in animal models of Parkinson's disease, and retrospective epidemiological studies indicate that they may reduce the risk to people of developing the disease, although the effects are modest (Stayte and Vissel 2014; Tansey and Goldberg 2010). The antibiotic minocycline, purportedly through an inhibitory action on microglia activity, is also neuroprotective in animal models of Parkinson's disease, but the effect has not been demonstrated to date in patients with Parkinson's disease (Stayte and Vissel 2014). Targeting inflammatory pathways other than cyclooxygenase derivatives, such as those that are modulated by proteinases and regulated by proteinase-activated receptors (PAR), might provide an alternative means of reducing neuroinflammation in brain (Bushell 2007).

Proteinases, in addition to hydrolysing peptide bonds between amino acids, also act as signalling molecules and have an important role in homeostatic regulation in mammals through actions on PAR, which act as sensors for the proteinases and also control their release through a feedback mechanism (Ramachandran et al. 2012). PAR belongs to the G-protein coupled seven transmembrane domain receptor family and is activated by cleavage of a section of the amino-terminus by serine proteinases to unmask a receptor-tethered ligand (i.e. the newly created amino-terminus). There are four subtypes; PAR1 and PAR3 are preferentially activated by thrombin, PAR2 is preferentially activated by trypsin and mast cell tryptase, and PAR4 is activated by both trypsin and thrombin. In addition, a number of other endogenous serine proteinases can activate each PAR (Vergnolle 2009). PAR2 has an established involvement in inflammatory cardiovascular, pulmonary and gastrointestinal diseases and may be involved in neuroinflammation seen in some neurodegenerative disorders, since increased PAR2 expression was found in the brain from

patients dying with Alzheimer's disease, multiple sclerosis and human immunodeficiency virus-associated dementia (Afkhani-Goli et al. 2007; Noorbakhsh et al. 2005, 2006; Rothmeier and Ruf 2012). However, the status of PAR2 in Parkinson's disease is unknown.

Recently, using a DNA microarray, significant alterations in messenger RNA (mRNA) for the serine proteinase trypsin-2 and two serine proteinase inhibitors (serpin-A5 and serpin-A13) were found in the substantia nigra of Parkinson's disease cases compared to controls, which suggested an involvement in the neurodegenerative process of Parkinson's disease, although the amount of mRNA for PAR2 itself was not significantly changed in the substantia nigra (Durrenberger et al. 2012). However, the mRNA was extracted from a tissue punch and would therefore represent a mixed population of cells, and opposite changes in expression in different cell types could mask changes. Consequently, the present study sought to quantify PAR2 protein expression, together with examination of the expression of two activator serine proteinases (trypsin-2, mast cell tryptase) and two serine proteinase inhibitors (serpin-A5, serpin-A13) of PAR2, in degenerating and non-degenerating brain regions at different Braak  $\alpha$ -synuclein stages of Parkinson's disease by immunohistochemistry and unbiased stereological cell counting techniques.

## Materials and Methods

### Post-mortem Human Brain

Slide-mounted formalin-fixed paraffin wax-embedded brain sections (6  $\mu$ m) from 14 control individuals, 29 patients diagnosed with Parkinson's disease and six cases found upon neuropathological examination to meet the diagnostic criteria for incidental Lewy body disease were obtained from the Parkinson's UK Tissue Bank at Imperial College via a prospective donor scheme with full ethical approval (80/MRE09/31+5). Not all brain regions were available from every case. Preliminary experiments used additional tissue with short post-mortem intervals obtained from the Brain and Body Donation Program, Arizona, USA, and found no significant effect of post-mortem interval on immunohistochemistry results (Supplementary Fig. S1). The cohorts did not differ for age, age at disease onset, disease duration or post-mortem interval (Table 1). Individual details for each case are provided in Supplementary Table S1. The clinical diagnosis of Parkinson's disease was confirmed post-mortem by neuropathological analysis, and the degree of  $\alpha$ -synuclein was rated and each case was allocated an  $\alpha$ -synuclein Braak stage (Alafuzoff et al. 2009; Braak et al. 2003). Control cases had no clinical diagnosis of a neurological or psychiatric disorder during life or any neuropathological abnormality evident post-mortem. The brain regions chosen for study ranged from those

**Table 1** Summary details of patients and control individuals who donated brain used in this study

Case	Age at death Years <sup>a</sup>	Gender M/F	Onset age Years <sup>b</sup>	Duration Years <sup>c</sup>	PMI Hours <sup>d</sup>	Alpha-synuclein Braak stage ( $n \times$ value)
Control						
14 cases	77±9 (58–93)	6/8	–	–	28±15 (9–52)	0
Incidental Lewy body disease						
6 cases	87±17 (58–104)	1/5	–	–	35±26 (10–74)	<2
Early Parkinson's disease						
10 cases	80±5 (72–87)	7/3	69±3 (66–77)	12±4 (6–18)	20±12 (5–45)	4× stage 3, 6× stage 4
Late Parkinson's disease						
19 cases	78±6 (61–87)	13/6	67±9 (47–80)	11±6 (2–23)	15±11 (3–42)	9× stage 5, 10× stage 6

Demographic details for donors who provided brain tissue. Values are mean±standard deviation with range in parentheses for age at death and disease onset, disease duration and post-mortem interval. Note that there is no relation between disease duration and severity which exemplifies the complicated aetiology of idiopathic Parkinson's disease

<sup>a,b,c,d</sup>Not significantly different between groups

with early involvement in the disease process that undergo profound cell loss and exhibit extensive  $\alpha$ -synuclein pathology (dorsal motor nucleus of the vagus, locus coeruleus, substantia nigra) to those with undergo little cell loss and only develop  $\alpha$ -synuclein inclusions at the end-stage of Parkinson's disease (anterior cingulate cortex, primary motor cortex). All Parkinson's disease cases received chronic treatment with L-DOPA (L-3,4-dihydroxyphenylalanine) and/or direct acting dopamine agonists prior to death.

### Immunohistochemistry

Immunohistochemistry was conducted using standard techniques as previously described (Hurley et al. 2013). Details of the antibodies used and experimental conditions are provided in Table 2. The antibodies for neuronal markers are all established experimental reagents purchased from reputable companies that have been used extensively in brain research. Preliminary experiments optimised the concentration of

**Table 2** Details of reagents used for immunohistochemistry

Antibody	Supplier	Type	Product number	Antigen retrieval <sup>a</sup>	Dilution
Primary					
P2A (PAR2)	A.F. Walls	Monoclonal—mouse	clone P2A	A	1:100
AA1 (mast cell tryptase)	A.F. Walls	Monoclonal—mouse	clone AA1	A	1:100
PRSS2 (trypsin-2)	Abcam	Polyclonal—mouse	AB76781	A	1:200
Serpin-A5 (proteinase inhibitor)	Abcam	Polyclonal—mouse	AB172060	B	1:200
Serpin-A13 (proteinase inhibitor)	Antibody Production Supplies	Polyclonal—rabbit	custom	A	1:175
Neuronal nuclei (neuN)	Millipore (Temecula)	Monoclonal—mouse	MAB377 (clone A60)	A	1:250
Tyrosine hydroxylase (TH)	Millipore (Temecula)	Polyclonal—rabbit	AB152	A	1:500
GFAP (astrocyte marker)	Abcam	Monoclonal—rabbit	AB33922	A	1:100
HLA-DR (activated microglia)	DAKO	Monoclonal mouse	M0775 (clone CR3/43)	B	1:200
IBA1 (microglia marker)	Abcam	Polyclonal—rabbit	AB108539	B	1:100
Secondary					
Biotin-SP-goat anti-rabbit	Stratech	IgG (H+L)	111-065-144	–	1:250
Biotin-SP-sheep anti-mouse	Stratech	IgG (H+L)	515-065-062	–	1:250
Alexa Fluor® 546 goat anti-rabbit	Invitrogen	IgG (H+L)	A11035	–	1:1000
Alexa Fluor® 488 goat anti-mouse	Invitrogen	IgG (H+L)	A11029	–	1:1000
Peroxidase conjugate					
Streptavidin-POD	Roche		12213700	–	1:250

Details of reagents used for immunohistochemistry. Monoclonal antibodies against PAR2 and mast cell tryptase were provided by Dr. Walls (Aslam et al. 2002; Walls et al. 1990a, b), and others were bought from reputable companies. The polyclonal antibody against serpin-A13 that was made to order by Antibody Production Services Ltd. using a synthetic peptide specific to serpin-A13 as the immunogen

<sup>a</sup>Thirty minutes in steamer in solution A (10 mM Tris, 1 mM EDTA, pH 9.2) or B (10 mM sodium citrate pH 6.1, 0.5 % Tween-20)

antibodies used and determined the best antigen retrieval technique for the available tissue. Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich. Briefly, sections were dewaxed, endogenous peroxidase was quenched and then antigens retrieved at high temperature and low or high pH. Non-specific sites were blocked with normal serum, and sections were incubated with primary antibody overnight. Sections were then incubated with a biotinylated secondary antibody followed by incubation with streptavidin peroxidase conjugate. Staining was visualised by incubation with diaminobenzidine, and for neuromelanin-containing brain sections, nickel sulphate was added to the diaminobenzidine solution. Sections were rinsed with water, cleared and coverslipped with Histoclear II and Omnimount (National Diagnostics) respectively.

For immunofluorescence, sections were processed similarly except that prior to application of the primary antibody combinations, sections were stained with Sudan Black B to reduce autofluorescence and goat anti-rabbit Alexa Fluor® 546 and goat anti-mouse Alexa Fluor® 488 (Invitrogen) were used as secondary antibodies, followed by incubation with DAPI (4',6-diamidino-2-phenylindole) to stain nuclei. Sections were then rinsed three times with water, coverslipped with fluorescent mounting medium (Vector Laboratories) and dried under pressure.

In all brain regions examined, staining was evident in perikarya and neuropil which was not present when primary antibody was omitted, and such staining was therefore considered to represent specific binding of the antibody to the respective protein in the tissue section.

### Image Acquisition

Light microscopic images of chromogen immunolabelled cells were captured with a 3-chip colour CCD digital camera (QIClick-F-CLR-12) attached to a Nikon Eclipse E800 microscope equipped with a motorised ( $x$ - $y$ -axis) stage using Image-Pro® Plus software v. 7.0 (Media Cybernetics, Rockville, MD, USA) with appropriate filters and light source. The same system was used to take photographs for the figures. Images of fluorescent dye-labelled cells were captured using a Leica TCS-SP5 II confocal scanning microscope and LAS AF imaging software v. 2.5.2.6939 (Leica). Adobe Photoshop Elements v. 9.0 was used to resize and adjust (automatically) the contrast and/or levels of images used in the figures of this manuscript.

### Cell Counting

Staining of cells with a cell body  $>15 \mu\text{m}$  and a multipolar morphology was considered to be neurons and small rounder cells with a diameter  $<10 \mu\text{m}$  were taken to be microglia.

An estimate of the total cell density was obtained by unbiased design-based stereological cell-counting techniques using a stereology add-in module of Image-Pro® Plus software. Results were presented as cells per square millimetre brain and not as a volume since a series of sections throughout each brain region were not available. Sections were tiled at  $\times 10$  magnification and a montage image created. The region of interest was delineated on this image and the counting area ( $A$ ) calculated using Cavalieri's principle and a  $350 \times 350\text{-}\mu\text{m}$  volume grid. Counting was conducted at  $\times 200$  magnification using a  $200 \times 200\text{-}\mu\text{m}$  systematic uniform random points experimental grid containing a  $125 \times 125\text{-}\mu\text{m}$  counting frame. All frames were counted. The number of cells in the region of interest was estimated using the formula:  $N = (1/\text{area sampling fraction}) \times \text{total cell count}$ , where the area sampling fraction is the area of the number of frames counted/ $A$ . The cell density (cells/ $\text{mm}^2$ ) in the region of interest was obtained by dividing the estimated cell number ( $N$ ) by the area of the region of interest for each case and then taking the mean across cases (Schmitz and Hof 2005).

### Data Analysis

Data (mean  $\pm$  SEM unless stated otherwise) were analysed using IBM SPSS™ (v. 22) statistics software and graphs plotted with GraphPad PRISM™ (v. 5). To determine differences between groups, cases were divided into control ( $n=12\text{--}13$ ), incidental Lewy body disease ( $\alpha$ -synuclein Braak stage  $<2$ ,  $n=5\text{--}6$ ), early Parkinson's disease ( $\alpha$ -synuclein Braak stage 3 or 4,  $n=9\text{--}10$ ) and late Parkinson's disease ( $\alpha$ -synuclein Braak stage 5 or 6,  $n=15$ ). The data were analysed for normalcy and then by an appropriate ANOVA and post hoc multiple comparison test. For correlation analysis, tests compared actual Braak  $\alpha$ -synuclein stage values for each case with the cell count values. Data from all groups were also analysed by ANCOVA using the full factorial univariate general linear model with the incidence of dyskinesia, psychosis and autonomic dysfunction as covariates. In addition, Parkinson's disease cases alone were also analysed by ANOVA and post hoc multiple-comparison test using the incidence of dyskinesia, psychosis or autonomic dysfunction as the independent variable.  $P < 0.05$  was considered significant.

### Results

The number of cells expressing PAR2, the activating serine proteinases trypsin-2 and mast cell tryptase, the serine proteinase inhibitors serpin-A5 and serpin-A13, and the cellular markers IBA1 (microglia), HLA-DR (activated microglia), neuN (neurons) and tyrosine hydroxylase (catecholamine neurons) were quantified using unbiased stereological counting techniques in five brain regions of control, incidental Lewy



body disease and early and late Braak  $\alpha$ -synuclein stage Parkinson's disease brain.

For PAR2, trypsin-2, serpin-A5 and serpin-A13 high levels (in comparison to the surrounding neuropil) of immunoreactivity were present in the soma and dendrites of neurons and in small rounder cells that were considered to be microglia in all brain regions examined (Fig. 1), whereas neuN and tyrosine hydroxylase staining was only detected in neurons and IBA1 and HLA-DR staining was only present on microglia (Fig. 1). Double labelling of sections and confocal immunofluorescent microscopy showed robust co-localisation of PAR2 with the microglial marker IBA1 but minimal signal with the marker for astrocytes (GFAP) (Fig. 2). Indicating that in aged brain non-neuronal PAR2 expression was predominantly in microglia.

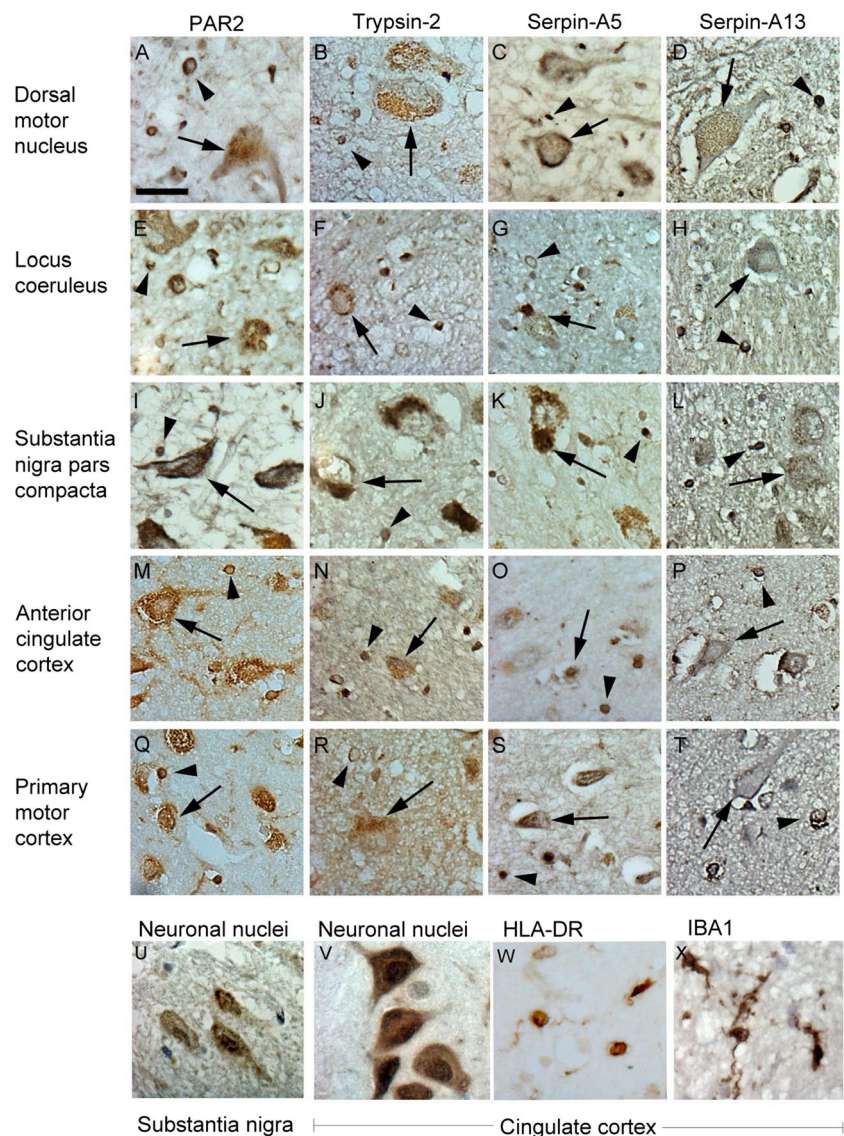
Cell count data for control brain are summarised for large cells ( $>15 \mu\text{m}$ ) with a multipolar morphology

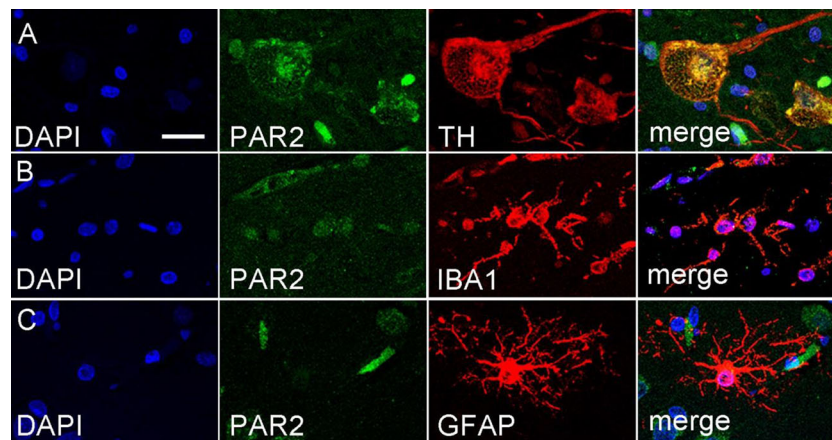
(neurons) and small rounder cells ( $<10 \mu\text{m}$ ) with microglial-like morphology (microglia) in Fig. 3. Data for incidental Lewy body disease and Parkinson's disease cases compared to controls are summarised in Fig. 4. Actual cell counts and correlation and ANCOVA results are given in Tables 3 and 4.

### Dorsal Motor Nucleus of the Vagus

**Neurons** The estimated density of neurons (neuN positive cells) in the dorsal motor nucleus of the vagus in control cases was  $187 \pm 17 \text{ cells/mm}^2$ , and there was no significant reduction in estimated neuron density in incidental Lewy body disease and Parkinson's disease cases. PAR2 and serpin-A13 were expressed in 59 and 60 % of neurons in the dorsal motor nucleus of the vagus, whereas 41, 25 and 17 % of neurons were positive for trypsin-

**Fig. 1** Representative staining of PAR2 (a, e, i, m, q), trypsin-2 (b, f, j, n, r), serpin-A5 (c, g, k, o, p) and serpin-A13 (d, h, l, p, t) in normal human brain. Arrows point toward an example of a neuron and arrowheads indicate microglia. Scale bar= $25 \mu\text{m}$ . The lower panel shows representative neuN staining of neurons in substantia nigra (u) and anterior cingulate cortex (v) and representative amoeboid and ramified microglia stained with HLA-DR (w) and IBA1 (x) respectively in anterior cingulate cortex. Scale bar= $25 \mu\text{m}$





**Fig. 2** Representative immunofluorescent staining of PAR2 and tyrosine hydroxylase (*TH*) in substantia nigra (**a**) and PAR2 and the microglia marker IBA1 (**b**), and the astrocyte marker GFAP (**C**) in anterior cingulate cortex of normal brain. Immunofluorescent double staining

for PAR2 (*green*) and IBA1 (microglia, *red*) or GFAP (astrocytes, *red*) showed that PAR2 was predominantly expressed by microglia not astrocytes in normal brain. Scale bar=25  $\mu$ m

2, serpin-A5 and tyrosine hydroxylase, respectively (Fig. 3). There was a significant negative correlation for a decrease in both serpin-A5 and serpin-A13 with increasing Braak  $\alpha$ -synuclein stage, and serpin-A5 expression was significantly reduced in late Parkinson's disease cases in comparison to control cases. The number of neurons expressing PAR2, trypsin-2 and tyrosine hydroxylase did not change significantly in the dorsal motor nucleus of the vagus of incidental Lewy body disease or Parkinson's disease cases (Fig. 4, Table 3).

**Microglia** The estimated number of microglia (IBA1-positive cells) in the dorsal motor nucleus of the vagus of control cases was  $212 \pm 12$  cells/mm<sup>2</sup>. Of these, the majority also expressed HLA-DR (82 %), trypsin-2 (77 %), serpin-A5 (86 %) and serpin-A13 (82 %), whereas only approximately half (47 %) of microglia expressed PAR2 (Fig. 3). With increasing Braak  $\alpha$ -synuclein stage, there was a significant positive correlation to increase the number of microglia stained with serpin-A5 and trypsin-2 and a significant negative correlation for a decrease in the number of microglia that expressed serpin-A13. The number of IBA1 stained cells increased in cases with early Parkinson's disease, and the number of microglia positive for serpin-A5 was significantly higher in the late-stage Parkinson's disease group (Fig. 4, Table 4).

#### Locus Coeruleus

**Neurons** The estimated density of neurons in the locus coeruleus of control cases was  $171 \pm 12$  cells/mm<sup>2</sup>, and there was a significant negative correlation for a decrease in neuronal density in cases with increased  $\alpha$ -synuclein pathology. Approximately half of the neurons co-

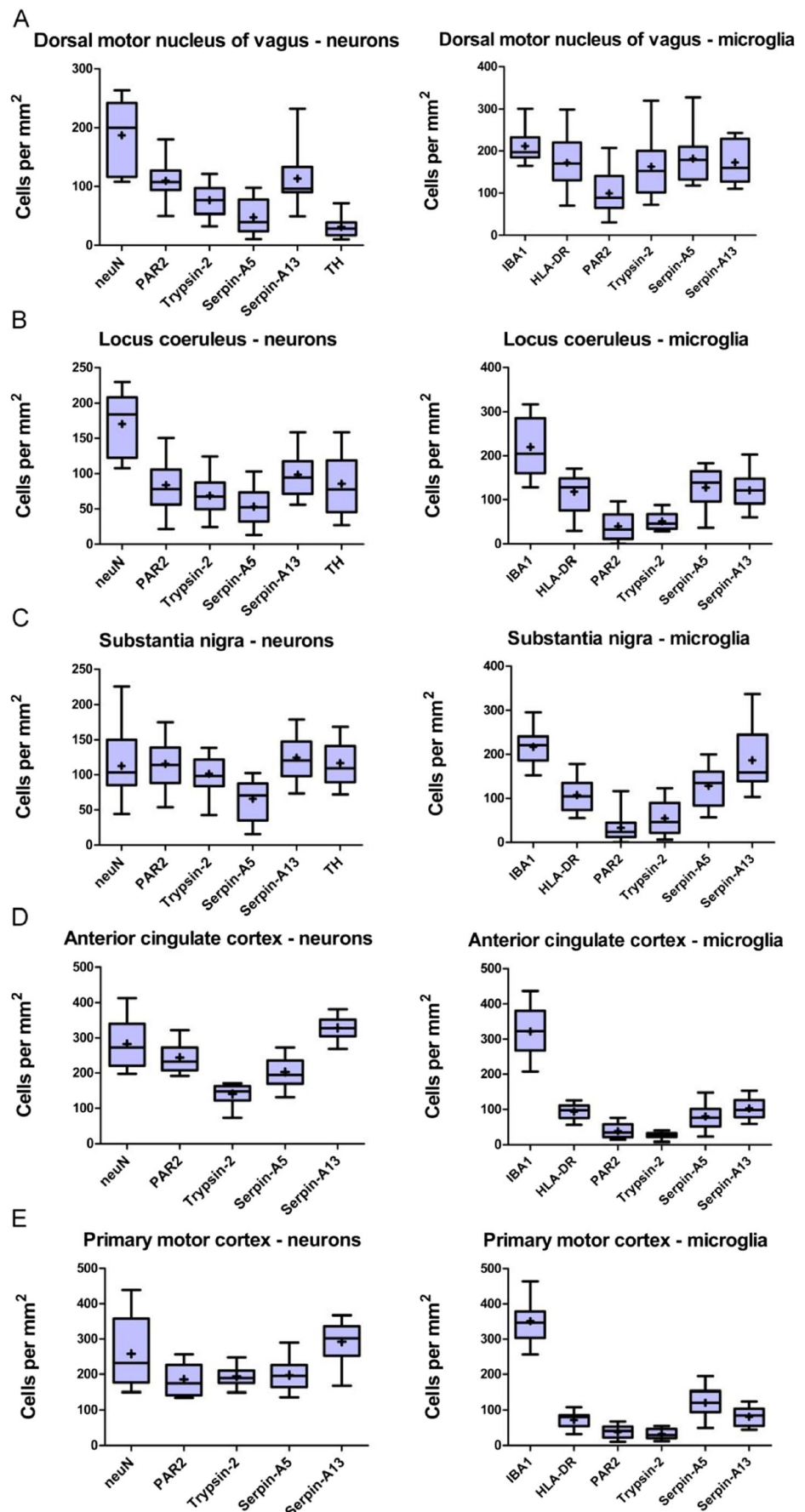
expressed PAR2 (49 %), serpin-A13 (57 %) and TH (50 %) in the locus coeruleus, whereas trypsin-2 and serpin-A5-positive neurons comprised 40 and 31 % of the total number of neurons, respectively (Fig. 3). There was a negative correlation for the expression of serpin-A13 and tyrosine hydroxylase with increasing Braak  $\alpha$ -synuclein stage, and both proteins were significantly reduced in late-stage Parkinson's disease cases. The number of neurons that expressed PAR2, trypsin-2 and serpin-A5 did not change significantly in cases with  $\alpha$ -synuclein pathology in comparison to control cases (Fig. 4, Table 3).

**Microglia** The estimated number microglia in the locus coeruleus of control cases was  $220 \pm 18$  cells/mm<sup>2</sup>. Of these, 83 % were HLA-DR positive, while less expressed serpin-A5 (58 %) and serpin-A13 (55 %). Trypsin-2 and PAR2 were only detected in 23 and 18 % of microglia, respectively (Fig. 3). The density of IBA1-positive microglia was significantly elevated in incidental Lewy body disease cases but not the Parkinson's disease groups. The number of microglia that expressed PAR2 showed a positive correlation to increase with advancing Braak  $\alpha$ -synuclein stage (Fig. 4, Table 4).

#### Substantia Nigra Pars Compacta

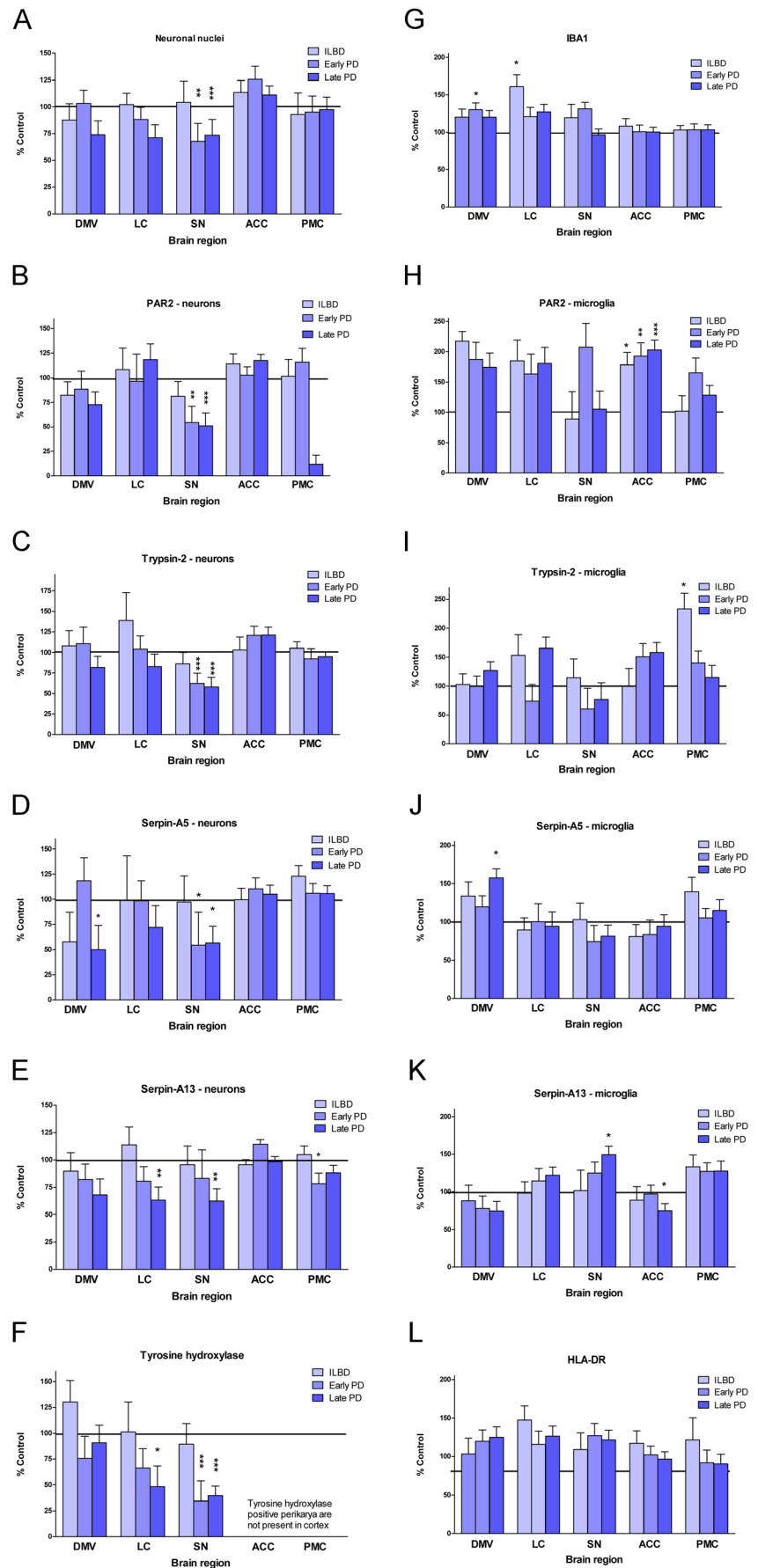
**Neurons** The estimated number of neurons in the substantia nigra was  $164 \pm 11$  cells/mm<sup>2</sup> in control cases and 71 % of these stained positive for both PAR2 and tyrosine hydroxylase, whereas serpin-A13-, trypsin-2- and serpin-A5-positive neurons represented 76, 62 and 40 % of the total neuronal population, respectively (Fig. 3). There was a significant negative correlation

**Fig. 3** Quantification of cell numbers in dorsal motor nucleus of the vagus (**a**), locus coeruleus (**b**), substantia nigra (**c**), anterior cingulate cortex (**d**) and primary motor cortex (**e**) of normal human brain for staining of neurons (*left panel*) with neuN, PAR2, trypsin-2, serpin-A5, serpin-A13 and tyrosine hydroxylase (*TH*) and of microglia (*right panel*) with IBA1, PAR2, trypsin-2, serpin-A5, serpin-A13 and HLA-DR. *Box* indicates 25th and 75th percentiles, *line* indicates median, *+* indicates mean, *whiskers* indicate 5th and 95th percentiles





**Fig. 4** Comparison of stereological cell counts for neurons (*left panel*) and microglia (*right panel*) from incidental Lewy body disease (*ILBD*), early Parkinson’s disease (*PD*) and late PD expressed as a percentage of the control value for staining with neuN (**a**), PAR2 (**b, g**), trypsin-2 (**c, i**), serpin-A5 (**d, j**), serpin-A13 (**e, k**), IBA1 (**g**), tyrosine hydroxylase (**f**) and HLA-DR (**l**). *DMV* dorsal motor nucleus of the vagus nerve, *LC* locus coeruleus, *SN* substantia nigra pars compacta, *ACC* anterior cingulate cortex, *PMC* primary motor cortex. The *line at 100 %* represents the control value. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$





**Table 3** Cell counts of neurons (cells/mm<sup>2</sup>)

	Control	ILBD (Braak 1 or 2)	Early Parkinson's (Braak 3 or 4)	Late Parkinson's (Braak 5 or 6)	Correlation	ANCOVA
Dorsal motor nucleus	<i>n</i> =12	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
Neuronal nuclei	187±17	164±20	193±16	139±13	No	None
PAR2	110±9	90±10	97±16	80±8	No	None
Trypsin-2	77±8	83±13	85±15	63±6	No	None
Serpin-A5	47±9	27±6	56±8	<b>24±4*</b>	<b>*-ve</b>	None
Serpin-A13	113±13	102±12	93±7	77±7	<b>*-ve</b>	None
Tyrosine hydroxylase	31±5	41±6	24±4	28±2	No	None
Locus coeruleus	<i>n</i> =13	<i>n</i> =5	<i>n</i> =10	<i>n</i> =15		
Neuronal nuclei	171±12	174±14	150±14	121±12	<b>*-ve</b>	None
PAR2	84±10	91±16	81±20	99±10	No	None
Trypsin-2	69±8	95±31	72±8	57±6	No	None
Serpin-A5	53±7	53±22	53±8	38±6	No	None
Serpin-A13	98±8	112±16	79±8 ( <i>n</i> =9)	<b>62±5**</b>	<b>**_-ve</b>	None
Tyrosine hydroxylase	86±12	87±22	57±7	<b>41±6*</b>	<b>**_-ve</b>	None
Substantia nigra	<i>n</i> =13	<i>n</i> =6	<i>n</i> =9	<i>n</i> =15		
Neuronal nuclei	164±11	163±15	<b>112±16**</b>	<b>97±8***</b>	<b>**_-ve</b>	None
PAR2	116±10	94±11	<b>63±9***</b>	<b>59±6***</b>	<b>**_-ve</b>	None
Trypsin-2	102±7	88±10	<b>63±7***</b>	<b>59±6***</b>	<b>**_-ve</b>	None
Serpin-A5	66±8	64±16	<b>36±11*</b>	<b>37±4*</b>	<b>**_-ve</b>	None
Serpin-A13	124±9	119±18	103±26	<b>78±7**</b>	<b>**_-ve</b>	None
Tyrosine hydroxylase	117±8	105±19	<b>40±7***</b>	<b>46±3***</b>	<b>**_-ve</b>	None
Ant. cingulate cortex	<i>n</i> =13	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
Neuronal nuclei	283±19	321±28	342±35	315±14	No	None
PAR2	244±12	279±25	251±18	287±11	<b>*+ve</b>	None
Trypsin-2	141±8	145±21	170±16	171±14	No	<b>Dyskinesia</b>
Serpin-A5	203±12	202±20	223±21	213±13	No	<b>Psychosis</b>
Serpin-A13	333±8 ( <i>n</i> =12)	314±13	370±12 ( <i>n</i> =9)	323±12	No	None
Primary motor cortex	<i>n</i> =13	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
Neuronal nuclei	258±26	237±41	245±27	248±16	No	None
PAR2	185±12	189±30	214±27	207±14	No	None
Trypsin-2	194±7	204±14	179±20	184±8	No	<b>Dyskinesia</b>
Serpin-A5	198±12	243±21	209±16	209±10	No	<b>Dyskinesia</b>
Serpin-A13	293±16	306±18	<b>229±19*</b>	258±11	<b>**_-ve</b>	<b>Dyskinesia</b>

Stereological cell counts of neurons. Immunoreactivity for neurons labelled with the neuronal nuclei marker, PAR2, trypsin-2, serpin-A5, serpin-A13 and tyrosine hydroxylase antibodies was quantified. Differences between groups (control, incidental Lewy body disease, early and late Parkinson's disease) were analysed by two-tailed ANOVA with post hoc multiple comparison. Changes where  $P < 0.05$  are in bold type. Cases were analysed by Braak  $\alpha$ -synuclein stage (0–6) for correlation analysis. Covariates for ANCOVA were dyskinesia, psychosis and autonomic dysfunction

ILBD incidental Lewy body disease

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

for a reduction in neurons that expressed all proteins examined with increasing Braak  $\alpha$ -synuclein stage, such that PAR2, trypsin-2, serpin-A5, neuN and TH-positive cell densities were all significantly reduced in comparison to control in both early and late-stage Parkinson's disease cases. Whereas, serpin-A13 was only significantly reduced in late-stage Parkinson's disease cases (Fig. 4, Table 3).

**Microglia** The estimated number of microglia in the substantia nigra of control cases was  $217 \pm 10$  cells/mm<sup>2</sup>, and this did not change significantly in the other experimental groups. The majority of microglia stained positive for HLA-DR (72 %), serpin-A13 (86 %) and serpin-A5 (59 %) in this brain region, whereas fewer microglia were positive for trypsin-2 (25 %) and PAR2 (15 %) (Fig. 3). The expression of PAR2, trypsin-2 and

**Table 4** Cell counts of microglia (cells/mm<sup>2</sup>)

	Control	ILBD (Braak 1 or 2)	Early Parkinson's (Braak 3 or 4)	Late Parkinson's (Braak 5 or 6)	Correlation	ANCOVA
Dorsal motor nucleus	<i>n</i> =12	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
IBA1	212±12	254±24	<b>276±19*</b>	254±18	No	<b>Autonomic</b>
HLA-DR	173±19	178±31	207±20	215±17	No	None
PAR2	99±15	216±15	186±44	173±31	No	None
Trypsin-2	163±21	168±22	162±21	208±16	<b>**+ve</b>	None
Serpin-A5	182±17	243±39	219±24	<b>287±22*</b>	<b>**+ve</b>	None
Serpin-A13	173±15	152±29	135±19	129±12	<b>*-ve</b>	None
Locus coeruleus	<i>n</i> =13	<i>n</i> =5	<i>n</i> =10	<i>n</i> =15		
IBA1	220±18	<b>353±49*</b>	265±25	280±16	No	None
HLA-DR	183±17	242±38	181±21	178±15	No	None
PAR2	40±9	73±18	65±15	71±9	<b>**+ve</b>	None
Trypsin-2	51±6	78±26	38±9	84±14	No	None
Serpin-A5	127±13	114±14	127±27	120±19	No	None
Serpin-A13	121±11	119±14	139±20 ( <i>n</i> =9)	148±10	No	None
Substantia nigra	<i>n</i> =13	<i>n</i> =6	<i>n</i> =9	<i>n</i> =15		
IBA1	217±10	259±38	227±20	209±14	No	None
HLA-DR	156±15	163±32	161±17	162±13	No	None
PAR2	33±9	29±13	56±18 ( <i>n</i> =8)	35±5	No	None
Trypsin-2	54±10	62±19	33±10	42±9	No	None
Serpin-A5	128±13	132±23	95±18	105±10	No	<b>Dyskinesia</b>
Serpin-A13	186±19	190±37	233±25	<b>279±14*</b>	<b>**+ve</b>	None
Ant. cingulate cortex	<i>n</i> =13	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
IBA1	304±18	348±28	324±22	316±10	No	None
HLA-DR	94±6	110±16	96±9 ( <i>n</i> =9)	91±7	No	None
PAR2	38±6	<b>67±9*</b>	<b>73±11**</b> ( <i>n</i> =9)	<b>77±5***</b>	<b>**+ve</b>	None
Trypsin-2	25±2	25±7	38±8	40±6	No	<b>Dyskinesia</b>
Serpin-A5	80±11	65±4	67±8	75±5	No	None
Serpin-A13	103±8	91±15	100±8	<b>77±4*</b>	<b>*-ve</b>	None
Primary motor cortex	<i>n</i> =13	<i>n</i> =6	<i>n</i> =10	<i>n</i> =15		
IBA1	352±17	362±12	363±23	356±18	No	None
HLA-DR	72±6	87±24	66±9	65±6	No	None
PAR2	38±5	39±9	62±13	48±5	No	None
Trypsin-2	33±4	<b>76±18**</b>	46±7	37±6	No	None
Serpin-A5	121±12	169±27	127±9	139±13	No	None
Serpin-A13	81±7	108±14	103±7	103±10	No	None
All regions						
Mast cell tryptase	<1	<1	<1	<1		
Toluidine blue	<1	<1	<1	<1		

Stereological cell counts of microglia. Immunoreactivity for microglia labelled with IBA1, HLA-DR, PAR2, trypsin-2, serpin-A5 and serpin-A13 antibodies were quantified. Differences between groups (control, incidental Lewy body disease, early and late Parkinson's disease) were analysed by two-tailed ANOVA with post hoc multiple comparison test. Changes where *P* = < 0.05 are in bold type. Cases were analysed by Braak α-synuclein stage (0–6) for correlation analysis. Covariates for ANCOVA were dyskinesia, psychosis and autonomic dysfunction

ILBD incidental Lewy body disease

\**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001

serpin-A5 did not change in diseased tissue, whereas serpin-A13 expression had a significant positive correlation with increasing Braak α-synuclein stage such that

the number of microglia that expressed serpin-A13 was significantly higher in late-stage Parkinson's disease cases (Fig. 4, Table 4).

## Anterior Cingulate Cortex

**Neurons** The estimated number of neurons in anterior cingulate cortex was  $283 \pm 19$  cells/mm<sup>2</sup> in control cases, and this was not significantly different in the diseased cases. All neurons expressed serpin-A13 (118 %), and the majority were positive for PAR2 (86 %) and serpin-A5 (72 %), whereas half expressed trypsin-2 (50 %) (Fig. 3). Note that the number of neurons positive for serpin-A13 was not significantly different to the number stained with the neuN antibody. There was a significant positive correlation for an increase in the number of neurons that expressed PAR2 with increasing Braak  $\alpha$ -synuclein stage, while expression of the remaining proteins remained constant (Fig. 4, Table 3).

**Microglia** The estimated number of microglia in anterior cingulate cortex of control cases was  $304 \pm 18$  cells/mm<sup>2</sup>, and a comparable amount of microglia were present in the other experimental groups. HLA-DR, serpin-A5 and serpin-A13 immunoreactivity was present in 31, 26 and 34 % of microglia, respectively, while PAR2 and trypsin-2 were only expressed by 12 and 8 % of microglia respectively in control cases (Fig. 3). PAR2 expression in microglia was significantly higher in all diseased cases in comparison to controls. There was a negative correlation in the number of microglia that expressed serpin-A13 with increasing Braak  $\alpha$ -synuclein stage, and the number of microglia that expressed serpin-A13 was decreased in late-stage Parkinson's disease cases (Fig. 4, Table 4).

## Primary Motor Cortex

**Neurons** In primary motor cortex, the estimated number of neurons was  $258 \pm 26$  cells/mm<sup>2</sup> in control cases, and there was no difference in total neuron number across experimental groups. PAR2, trypsin-2, serpin-A5 and serpin-A13 were expressed in 72, 75, 77 and 119 % of neurons, respectively (Fig. 3). Again, the number of neurons positive for serpin-A13 was not significantly different to the number stained with the neuN antibody. There was a significant negative correlation for decreased neuronal expression of serpin-A13 with increasing Braak  $\alpha$ -synuclein stage, and serpin-A13 was decreased in the early Parkinson's disease group (Fig. 4, Table 3).

**Microglia** The estimated number of microglia in primary motor cortex of control cases was  $352 \pm 17$  cells/mm<sup>2</sup>, and a comparable amount of microglia were counted in the experimental groups with  $\alpha$ -synuclein pathology. A third of the microglia were positive for serpin-A5 (34 %), whereas serpin-A13 and HLA-DR comprised 23 and 20 % of microglia, respectively. Fewer microglia stained for PAR2 (11 %) and trypsin-2 (9 %) in primary motor cortex (Fig. 3). Trypsin-2 expression significantly increased in microglia of incidental Lewy body disease

cases. There was no difference in cell counts for the immunoreactivity of other antibodies between control and diseased tissue (Fig. 4, Table 4).

## Mast Cell Tryptase

Staining for mast cell tryptase was limited to very few cells per entire section (Table 4). Positive controls (brain sections with an active multiple sclerosis lesion and tonsil) indicated that the antibody and staining technique worked properly, and similar results were obtained using toluidine blue that chemically stains proteoglycans in mast cells (data not shown). More mast cell tryptase-positive cells were apparent in brain from young (<40 years) donors, but they were not quantified since tissue was only available from two cases.

## Analysis of Covariance

**Dorsal Motor Nucleus of the Vagus** The covariate autonomic dysfunction had a significant effect on the adjusted means of cell counts for IBA1 in the dorsal motor nucleus. However, this did not affect the outcome of the ANOVA, indicating that there was an interaction, but it was not strong enough to create a significant difference between the experimental cohorts (Table 4).

**Locus Coeruleus** None of the covariates included in the ANCOVA had a significant effect on the adjusted means for the cell counts for any of the proteins examined in the locus coeruleus.

**Substantia Nigra Pars Compacta** The covariate dyskinesia had a significant effect on the adjusted means for serpin-A5-labelled microglia in the substantia nigra. However, this did not affect the outcome of the ANOVA, indicating that there was an interaction, but it was not strong enough to create a significant difference between the experimental cohorts (Table 4).

**Anterior Cingulate Cortex** In anterior cingulate cortex, the covariate dyskinesia had a significant effect on the adjusted means for trypsin-2-labelled neurons and microglia, and the covariate psychosis had a significant effect on neurons labelled with serpin-A5. However, these effects did not affect the outcome of the ANOVA, indicating that there was an interaction but it was not strong enough to create a significant difference between the experimental cohorts (Tables 3 and 4).

**Primary Motor Cortex** In primary motor cortex, the covariate dyskinesia had a significant effect on the adjusted means for neurons labelled with serpin-A5, serpin-A13 and trypsin-2. However, these effects did not affect the outcome of the ANOVA, indicating that there was an interaction but it was not

strong enough to create a significant difference between the experimental cohorts (Table 3).

The ANCOVA interactions were found when the degree of dyskinesia, psychosis and autonomic dysfunction were included as covariates prompted analysis of just the Parkinson’s disease cases, in order to investigate the effect of the reported incidence of L-DOPA-induced side effects (dyskinesia, psychosis) and of autonomic dysfunction on the expression of PAR2, trypsin-2, serpin-A5 and serpin-A13.

**Dyskinesia** In the locus coeruleus, serpin-A5 expression was reduced in neurons and serpin-A13 expression increased in microglia in cases with a low incidence of dyskinesia compared to cases with no reported motor complications. In substantia nigra, serpin-A5 expression was significantly higher in microglia in cases with a high incidence of dyskinesia. In anterior cingulate cortex, trypsin-2 expression was significantly lower in microglia of high dyskinesia cases. In primary motor cortex, trypsin-2 and serpin-A13 were expressed in significantly fewer neurons in cases with high levels of dyskinesia, and there were significantly less serpin-A5-positive neurons in cases with a low incidence of dyskinesia (Table 5).

**Psychosis** In the locus coeruleus and substantia nigra, serpin-A5 expression was increased in microglia in cases with a low incidence of psychosis compared to cases with no reported psychiatric disturbance. In anterior cingulate cortex, significantly less trypsin-2-positive microglia were detected in cases with a high incidence of psychosis. In primary motor cortex, serpin-A5-positive microglia were increased in cases with a high incidence of psychosis, and trypsin-2-positive microglia were increased in cases with low and high levels of psychosis compared to cases with no reported psychiatric problems (Table 5).

**Autonomic** No differences in staining for any of the antibodies were found when Parkinson’s disease cases were analysed with respect to the amount of autonomic dysfunction.

**Discussion**

This study examined the expression of PAR2, the serine proteinases trypsin-2 and mast cell tryptase and two serine proteinase inhibitors (serpin-A5 and serpin-A13) in post-mortem human brain by immunohistochemistry with quantification using an unbiased stereological counting technique. Note that when counting, a cell was considered positive if staining was darker than the surrounding neuropil. Thus, decreased expression to background levels would result in a reduced cell count, in the absence of actual cell loss. Reduced cell counts can therefore arise from actual cell loss (e.g. dead dopamine

**Table 5** Summary of significant differences between cell counts (cells/mm<sup>2</sup>) for Parkinson’s disease cases when analysed using dyskinesia or psychosis as the independent variable

	Dyskinesia incidence		
	None	Low	High
Locus coeruleus	<i>n</i> =5	<i>n</i> =7	<i>n</i> =11
Serp-A5—neurons	63±11	<b>27±9*</b>	47±7
Serp-A13—microglia	117±23	<b>171±16*</b>	144±12
Substantia nigra	<i>n</i> =7	<i>n</i> =4	<i>n</i> =13
Serp-A5—microglia	63±8	109±29	<b>119±11**</b>
Anterior cingulate cortex	<i>n</i> =6	<i>n</i> =7	<i>n</i> =12
Trypsin-2—microglia	59±8	50±8	<b>23±3**</b>
Primary motor cortex	<i>n</i> =6	<i>n</i> =8	<i>n</i> =12
Trypsin-2—neurons	222±21	181±11	<b>165±13*</b>
Serp-A5—neurons	246±15	<b>195±19*</b>	199±11
Serp-A13—neurons	249±15	253±18	<b>217±14*</b>
	Psychosis incidence		
	None	Low	High
Locus coeruleus	<i>n</i> =5	<i>n</i> =4	<i>n</i> =15
Serp-A5—microglia	80±24	<b>216±30**</b>	117±19
Substantia nigra	<i>n</i> =6	<i>n</i> =4	<i>n</i> =14
Serp-A5—microglia	66±9	<b>146±15**</b>	103±12
Anterior cingulate cortex	<i>n</i> =6	<i>n</i> =4	<i>n</i> =15
Trypsin-2—microglia	56±8	39±13	<b>33±5*</b>
Primary motor cortex	<i>n</i> =6	<i>n</i> =4	<i>n</i> =14
Serp-A5—microglia	98±11	148±17	<b>144±12*</b>
Trypsin-2—microglia	19±6	<b>63±8**</b>	<b>44±6*</b>

Summary of significant differences (bold type) between Parkinson’s disease cases when analysed using the incidence of dyskinesia or psychosis as the independent variable. There were no differences between cases when the incidence of autonomic dysfunction was used as the independent variable

\**P*<0.05; \*\**P*<0.01

neuron) and decreased expression below the detection threshold. Likewise, increased expression could result in an increase in apparent cell number without actual neurogenesis or proliferation for neurons and glia respectively having occurred.

For neurons, the overall pattern was for a decrease in neurons positive for proteinase inhibitors (serpin-A5 and serpin-A13) with increasing Braak  $\alpha$ -synuclein stage in the dorsal motor nucleus, locus coeruleus and primary motor cortex that indicated decreased expression in neurons in these areas, since the total density of neurons did not significantly decrease in these regions. Although for the locus coeruleus, the number of tyrosine hydroxylase-positive (noradrenergic) neurons did significantly decrease, which is in keeping with published data (German et al. 1992; Hurley et al. 2013; Paulus and Jellinger 1991). In the substantia nigra, all proteins examined were significantly decreased, and this reflected the profound loss of tyrosine hydroxylase-positive (dopaminergic) neurons in this brain region. In contrast, in anterior cingulate cortex,



PAR2 immunoreactivity had a significant trend to increase with a higher Braak  $\alpha$ -synuclein stage rating.

With regard to microglia, where changes in expression occurred for the serpins (in dorsal motor nucleus of vagus and substantia nigra), trypsin-2 (in dorsal motor nucleus of vagus and primary motor cortex) and PAR2 (in locus coeruleus and anterior cingulate cortex), it reflected an increase in the number of microglia that expressed the protein that correlated with advancing Braak  $\alpha$ -synuclein stage and hence Parkinson's disease severity.

This study is the first description of PAR2, trypsin-2 and serpin expression in Parkinson's disease and provides evidence of altered PAR2 homeostasis in the disorder. Whether these changes are an intrinsic part of the disease process or an epiphenomenon, the putative altered PAR2 signalling is amenable to correction by drug treatment with a PAR2 antagonist. This view is supported by the recent study by Liu and colleagues (2014) who showed that antagonism of PAR2 could reverse behavioural deficits in a rodent model of Parkinson's disease.

Our data has parallels with that of Afkhami-Goli and colleagues (2007) who examined PAR2 expression in post-mortem brain from patients dying with Alzheimer's disease and found decreased PAR2 expression in neurons and also that PAR2-mediated activation of glial cells in mice exacerbated neurodegeneration. Likewise, Noobakhsh and colleagues (2006) found PAR2 immunoreactivity was increased in glial cells in white matter in brain from patients dying with multiple sclerosis, whereas neuronal PAR2 expression was unchanged.

### What Is the Function of PAR2 and Proteinases in Brain?

Neuronal PAR2 is believed to act as sensors for proteinases and regulates proteinase activity through a feedback mechanism that controls release of proteinases, thereby regulating extracellular processes that are involved in synaptic plasticity (Bushell 2007; Lohman et al. 2008; Wang et al. 2008). A number of studies have demonstrated that PAR2 and proteinases that can activate it are involved in synaptogenesis, long-term potentiation/depression and contribute to excitotoxicity through cleavage of the NR1 subunit of NMDA glutamate receptors (Hoffman et al. 1998; Nicole et al. 2001; Qian et al. 1993; Scarisbrick et al. 2001; Tomimatsu et al. 2002; Yepes et al. 2002), which together indicate that PAR2 signalling has a neuromodulatory role in brain.

The involvement of PAR2 and serine proteinases in long-term potentiation and depression is particularly interesting given the altered trypsin-2 and serpin expression levels in cases with dyskinesia, since inappropriate long-term potentiation and depression, together with altered dendritic spine

density, are thought to underlie treatment-induced dyskinesia in Parkinson's disease (Jenner 2008). Consequently, this raises the intriguing possibility that PAR2 antagonists might be useful in the treatment of L-DOPA and dopamine agonist-induced dyskinesia and psychosis in addition to the putative neuroprotective role due to their anti-inflammatory action. It is interesting to note that amantadine, through a presumed antagonism of NR1 glutamate receptors, is the only drug that has a proven anti-dyskinetic action in humans (Jenner 2008; Rodnitzky and Narayanan 2014).

### Serpins

Serine proteinase inhibitors (serpins) tightly regulate the activity of serine proteinases, to prevent detrimental uncontrolled proteolysis. Serpins comprise a large superfamily of proteins that control processes that require tight regulation such as blood coagulation, inflammation and fibrinolysis (Huntington 2011). But, their role in brain function is unknown, although they have been implicated in multiple sclerosis (serpin-A5), Alzheimer's disease (serpin-A3) and neuronal plasticity (neuroserpin) (Han et al. 2008; Hastings et al. 1997; Janciauskiene and Wright 1998). Based on the findings of this study, it would appear that they also have altered activity in Parkinson's disease.

### Mast Cells

Mast cells are a first line of defence alongside microglia in the innate immune system. In vitro, mast cell tryptase can activate microglia via PAR2 and PAR2 expression on mast cells that can be increased by tumour necrosis factor (Zhang et al. 2010, 2012). There were very low numbers of mast cells in the brain regions examined in this study. This was not wholly unexpected, since in humans, mast cells are most abundant in young individuals and their density in brain declines with age (Turygin et al. 2005). Furthermore, the few studies that have measured mast cell numbers in brain count them in *thousands per whole brain* (Silver and Curley 2013). However, the scarcity of mast cells in brain does not mean that their physiological role is minor, since the mediators they release can have effects over distances of more than 50  $\mu$ m from the cell of origin (Silver and Curley 2013). The significance of the age-related decline in mast cells is unknown but could relate to the immunosenescence that occurs with age (Gunin et al. 2011).

### Conclusions

In summary, this study showed for the first time that there was altered expression of PAR2 and some proteins that can control

PAR2 function in Parkinson's disease compared to age-matched controls. The changes measured in this study are not necessarily dependent upon each other, since trypsin-2 could be acting on other PAR subtypes and the serpins could be inhibiting a number of proteinases. However, if these changes reflect the process of neuroinflammation, then modulation of PAR2 activation with drugs might have a beneficial effect on the progression of Parkinson's disease. Further investigation of the role of PAR2 in Parkinson's disease and animal models of the disorder is warranted.

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**Conflict of Interest** The authors declare that they have no conflicts of interest.

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