Altered brain expression of proteinase-activated receptor-2 and associated proteinases and inhibitors in Parkinson's disease

Michael J. Hurley¹, Pascal F. Durrenberger¹, Steve M. Gentleman², Andrew F. Walls³ and David T. Dexter¹

¹Centre for Neuroinflammation and Neurodegeneration, Division of Brain Sciences, Department of Medicine, Imperial College London, Hammersmith Campus, Du Cane Road, London, W12 0NN, UK.

²Neuropathology Unit, Division of Brain Sciences, Department of Medicine, Imperial College London, Charing Cross Campus, St Dunstan's Road, London, W6 8RP, UK.

³Immunopharmacology Group, University of Southampton, Southampton General Hospital, Southampton, SO16 6YD, UK.

Correspondence to: Michael Hurley, Centre for Neuroinflammation and Neurodegeneration, Division of Brain Sciences, Department of Medicine, Hammersmith Campus, Du Cane Road, Imperial College, London, W12 0NN, UK.

e-mail: m.hurley@imperial.ac.uk, Tel. +44 207 589 5111; Fax +44 207 594 6548

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Abstract

In Parkinson's disease neuroinflammation is considered an important mechanism leading to bystander neuronal cell death, but the factors involved in the inflammatory process are not completely understood. Proteinase-activated receptor-2 is involved in neuroinflammation occurring in Alzheimer's disease and multiple sclerosis, but the status of proteinase-activated receptor-2 in Parkinson's disease is unknown. This study examined the expression of proteinase-activated receptor-2 and endogenous activators (trypsin-2, mast cell tryptase) and inhibitors (serpin-A5, serpin-A13) of the receptor in areas vulnerable and resistant to neurodegeneration in Parkinson's disease at different Braak α -synuclein stages of the disease in post-mortem brain.

In neurologically normal brain expression of proteinase-activated receptor-2, activating proteinases and proteinase inhibitors was found in neurons and microglia and did not specifically associate with regions prone to neurodegeneration in Parkinson's disease. In Parkinson's disease brain, alterations in the amount of immunoreactivity for proteinase-activated receptor-2, trypsin-2 and both serpin proteinase inhibitors were found throughout the brain. In neurons, there was a decrease in neurons positive for proteinase inhibitors with increasing Braak α -synuclein stage in the dorsal motor nucleus, locus coeruleus, substantia nigra and primary motor cortex. In contrast, in the cingulate cortex, proteinase-activated receptor-2 expression had a positive correlation to increase with higher Braak α -synuclein stage rating and serpin-A13 was increased in early Parkinson's disease cases compared to controls. In microglia, increased expression occurred for the serpins (dorsal motor nucleus of vagus and substantia nigra), trypsin-2 (dorsal motor nucleus of vagus and primary motor cortex) and proteinase-activated receptor-2 (locus coeruleus and cingulate cortex) that progressively increased with advancing Parkinson's disease severity.

In cingulate cortex, the covariate dyskinesia had a significant effect on the the adjusted means for trypsin-2 labelled neurons and microglia and the covariate psychosis had a significant effect on neurons labelled with serpin-A5. While in primary motor cortex, the covariate dyskinesia had a significant effect on the the adjusted means for neurons labelled with serpin-A5, serpin-A13 and trypsin-2. Analysis of Parkinson's disease cases found that serpin and trypsin-2 expression in midbrain and cerebral cortex was different in cases with dyskinesia and psychosis compared to those with no treatment induced side-effects.

This study showed that there was altered expression in brain of proteinase-activated receptor-2 and some proteins that can control its function in Parkinson's disease. Given the role of

proteinase-activated receptor-2 in neuroinflammation, drugs that mitigate these changes could be neuroprotective when administered to patients with Parkinson's disease.

Keywords: Proteinase-activated receptor-2, serine proteinase inhibitor clade A, Parkinson's disease, neuroinflammation, dyskinesia

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, it is characterized by degeneration of brainstem nuclei and the accumulation of α -synuclein-containing 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 which 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 in the caudate nucleus and putamen or directly stimulate dopamine receptors and while effective in restoring motor function, they have little effect on non-motor symptoms 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 neuroinflammation is thought to contribute to the neurodegenerative process (Barnum and Tansey, 2012; McGeer et al., 1988). Anti-inflammatory compounds are neuroprotective in animal models of Parkinson's disease and retrospective epidemiological studies indicate they may reduce the risk to people of developing the disease (Tansey and Goldberg, 2010). However, the effect of non-steroidal anti-inflammatory drugs and minocycline (through an action that inhibits microglia activity) are modest and the result of a clinical trial with minocycline was disappointing (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 ameliorating neuroinflammation in brain. 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 which are both dependent on proteinases and their target receptors (Kettenmann et al., 2011).

In addition to hydrolyzing peptide bonds between amino acids, proteinases act as signaling molecules that play an important role in homeostatic regulation in mammals through actions

on PAR, which act as sensors for the proteinases and control their release through a feedback mechanism (Ramachandran et al., 2012). PAR belong to the G-protein coupled seven transmembrane domain receptor family and are 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 preferentially activated by trypsin and thrombin, as well as a number of other endogenous serine proteinases that can activate (and inactivate) each PAR (Vergnolle, 2009). PAR2 has an established involvement in inflammatory cardiovascular, pulmonary and gastrointestinal diseases and is also involved in neuroinflammation seen in some neurodegenerative disorders, since increased PAR2 expression was found in brain in Alzheimer's disease and multiple sclerosis (Afkhani-Goli et al., 2007; Noorbakhsh et al., 2006; Rothmeier and Ruf, 2012). However, the status of PAR2 in Parkinson's disease is unknown.

Recently, using a DNA microarray, we found significant alterations in mRNA for the serine proteinase trypsin-2 and two serine proteinase inhibitors (serpin-A5 and serpin-A13) in the substantia nigra of Parkinson's disease cases compared to controls, which suggested an involvement of PAR2 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 α -synuclein stages of Parkinson's disease by immunohistochemistry and stereological cell counting.

Materials and Methods

Post-mortem human brain

Slide-mounted formalin-fixed paraffin wax embedded brain sections (6 μ m) from fourteen control individuals, twenty-nine 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). Note that not all brain regions were available from all cases. The cohorts did not differ for age, age at disease onset, disease duration or post-mortem interval and summary demographic data is presented in Table 1. Full individual demographic data can be found in supplementary Table 1.

Preliminary experiments showed that the post-mortem interval did not affect the signal of PAR2 in brain (Supplementary Fig 1). The clinical diagnosis of Parkinson's disease was confirmed post-mortem by neuropathological analysis and the degree of α -synuclein was rated and each case was allocated an α -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, nor any neuropathological abnormality evident post-mortem. The brain regions chosen for study ranged from those with early involvement in the disease process which undergo cell loss and exhibit extensive α -synuclein pathology to those which do not undergo cell loss and only contain α -synuclein inclusions at the end-stage of Parkinson's disease. 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

Details of the antibodies used, their dilutions and supplier are provided in Table 2. Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK). Immunohistochemistry was conducted using standard techniques as previously described (Hurley et al., 2013). 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 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 nickel sulphate and then sections were rinsed with water and cleared and coverslipped.

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 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 and dried under pressure.

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

Cell counting and data analysis

Stereology and image acquisition was conducted as previously described using a Nikon Eclipse E800 microscope equipped with a motorized (x-y axis) stage and Image-Pro® Plus stereology software v. 7.0 (Media Cybernetics, Rockville, MD, USA) (Hurley et al., 2013). 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) was used to resize and adjust (automatically) the contrast and/or levels of images used in the figures of this manuscript.

Data were analysed using IBM SPSS™ (v.19) statistics software. Values are mean $\pm$ SEM unless stated otherwise. For analysis cases were divided into control (n = 12-13), incidental Lewy body disease (α -synuclein Braak stage < 2, n = 5-6), early Parkinson's disease (α -synuclein Braak stage 3 or 4, n = 9-10) and late Parkinson's disease (α -synuclein Braak stage 5 or 6, n = 15). The data were analysed for normalcy and then analysed by an appropriate ANOVA and post-hoc multiple comparison tests. For correlation analysis, tests compared actual Braak α -synuclein stage values for each case with the cell counts. Data were also analysed by full factorial univariate general linear model ANCOVA using the incidence of dyskinesia, psychosis and autonomic dysfunction as covariates. Parkinson's disease cases were also analysed by ANOVA and post-hoc multiple-comparison test using the incidence of dyskinesia or psychosis or autonomic dysfunction as the independent variable. $P < 0.05$ was considered significant.

Results

The number of cells expressing PAR2, the activating proteinases trypsin-2 and mast cell tryptase, the proteinase inhibitors serpin-A5 and serpin-A13, IBA1 (microglia), HLA-DR (activated microglia), neuN (neurons) and tyrosine hydroxylase (catecholamine neurons) was quantified by stereology in five brain regions of control, incidental Lewy body disease and early and late Braak α -synuclein stage Parkinson's disease brain.

The immunohistochemical staining revealed that high levels (in comparison to the surrounding neuropil) of PAR2, trypsin-2, serpin-A5 and serpin-A13 was present in the soma and dendrites of neurons in all brain regions of normal brain and that the antibodies stained similar populations of neurons in each brain region (Figure 1). For PAR2 this was confirmed by double-labelling of the sections and confocal immunofluorescent microscopy (Figure 2). In addition, staining for these antibodies was evident in small cells which were considered to be microglia (Figure 1), since we did not find overlap of PAR2 staining with a marker for astrocytes (GFAP), but did observe co-localization with the microglia marker IBA1 (Figure 2).

Cell count data are summarized in Tables 3 and 4. For counting cells were divided into two groups. Namely, large cells with a multipolar morphology (neurons) and small rounder cells with microglial like morphology (microglia).

Dorsal motor nucleus of the vagus

The estimated density of neurons (neuN positive cells) in the dorsal motor nucleus of the vagus in control cases was 187 ± 17 cells/mm² 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 61% of neurons in the dorsal motor nucleus of the vagus, while 41%, 25% and 17% of neurons were positive for trypsin-2, serpin-A5 and TH respectively. There was a significant negative correlation for a decrease in both serpin-A5 and serpin-A13 with increasing Braak α -synuclein stage and serpin-A5 expression was significantly reduced by 50% in late Parkinson's disease cases in comparison to control cases. The number of neurons expressing PAR2, trypsin-2 and TH did not change significantly in the dorsal motor nucleus of the vagus of incidental lewy body or Parkinson's disease cases (Table 3).

The estimated number of microglia (IBA1 positive cells) in the dorsal motor nucleus of the vagus of control cases was 212 ± 12 cells/mm². Of these, 82% expressed HLA-DR, 77% expressed trypsin-2, 86% expressed serpin-A5 and 82% expressed serpin-A13. Whereas, only 47% of microglia expressed PAR2. With increasing Braak α -synuclein stage, there was a significant positive correlation to increase the number of microglia positive for 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 microglia increased by 30% in cases with early

Parkinson's disease and the number of microglia positive for serpin-A5 was significantly higher (58%) in the late stage Parkinson's disease cohort (Table 4).

The covariate autonomic dysfunction had a significant effect on the 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

The estimated density of neurons in the locus coeruleus of control cases was 171 ± 12 cells/mm² and there was a significant negative correlation for a decrease in neuronal density with increasing amounts of α -synuclein pathology. Approximately half of the neurons co-expressed PAR2 (49%), serpin-A13 (58%) 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. There was a negative correlation for the expression for serpin-A13 and TH with increasing Braak α -synuclein stage and serpin-A13 and TH 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 α -synuclein pathology in comparison to control cases, despite the trend for a reduction in the total number of neurons, which indicated that the neurons that expressed these proteins did not degenerate (Table 3).

The estimated number microglia in the locus coeruleus of control cases was 220 ± 18 cells/mm². Of these, 83% were HLA-DR positive and a similar number expressed serpin-A5 (58%) and serpin-A13 (55%), whereas trypsin-2 and PAR2 were only expressed in 23% and 18% of microglia respectively. The density of microglia was significantly elevated by 60% 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 increasing Braak α -synuclein stage (Table 4).

None of the covariates included in the ANCOVA had a significant effect on the the adjusted means for the cell counts for any of the antibodies.

Substantia nigra pars compacta

The estimated number of neurons in the substantia nigra was 164 ± 11 cells/mm² in control cases. PAR2 and TH positive neurons comprised 70% and 71% respectively of the total number of neurons in the substantia nigra while serpin-A13, trypsin-2 and serpin-A5 positive

neurons represented 76%, 62% and 40% of the total neuronal population respectively. There was a significant negative correlation for a reduction in neurons expressing all proteins examined with increasing Braak α -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 (Table 3).

The estimated number of microglia in the substantia nigra of control cases was 217 ± 10 cells/mm² and the majority stained positive for HLA-DR (72%), serpin-A13 (86%) and serpin-A5 (59%) in this brain region. Fewer microglia were positive for trypsin-2 (25%) and PAR2 (15%). The expression of PAR2, trypsin-2 and serpin-A5 did not change in diseased tissue, whereas serpin-A13 expression had a significant positive correlation with increasing Braak α -synuclein stage. The number of microglia that expressed serpin-A13 was significantly higher in late stage Parkinson's disease cases (Table 4).

The covariate dyskinesia had a significant effect on the the adjusted means for PAR2 and 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).

Cingulate cortex

The estimated number of neurons in cingulate cortex was 283 ± 19 cells/mm² in control cases and this did not change in the diseased cases. All neurons expressed serpin-A13 (116%) and the majority were positive for PAR2 (86%) and serpin-A5 (72%), while half expressed trypsin-2 (50%). There was a significant positive correlation for an increase in the number of neurons that expressed PAR2 with increasing Braak α -synuclein stage and the number of cells positive for serpin-A13 increased by 14% in early Parkinson's disease cases (Table 3).

The estimated number of microglia in the cingulate cortex of control cases was 304 ± 18 cells/mm² and a comparable amount of microglia were counted in the diseased tissue experimental groups. HLA-DR, serpin-A5 and serpin-A13 immunoreactivity was present in 31%, 26% and 34% of microglia respectively. Whereas, PAR2 and trypsin-2 were only expressed by 12% and 8% of microglia respectively. There was a significant positive correlation for an increase in the number of microglia that expressed PAR2 and 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 α -synuclein stage and the number of microglia that expressed serpin-A13 decreased in late stage Parkinson's disease cases (Table 4).

In cingulate cortex, the covariate dyskinesia had a significant effect on the 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 estimated number of neurons was 258 ± 26 cells/mm² 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 and their expression did not change in any of the diseased tissue groups. There was a significant negative correlation for decreased neuronal expression of serpin A13 with increasing Braak α -synuclein stage (Table 3).

The estimated number of microglia in primary motor cortex of control cases was 352 ± 17 cells/mm² and a comparable amount of microglia were counted in the experimental groups with α -synuclein pathology. A third of the microglia were positive for serpin-A5 (34%) while 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. Trypsin-2 expression, significantly increased in microglia of incidental Lewy body disease cases. There was no difference in cell counts for the other antibodies between control and diseased tissue (Table 4).

In primary motor cortex, the covariate dyskinesia had a significant effect on the 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.

Mast cell tryptase

Staining for mast cells tryptase was limited to very few cells per entire section (Table 4). Positive controls (sections with an active multiple sclerosis lesion and tonsil) indicated that the antibody and staining technique worked properly. Similar results were obtained using toluidine blue which chemically stains proteoglycans in mast cells. 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.

L-DOPA-induced side-effects

The ANCOVA interactions found when dyskinesia and psychosis were included as covariates prompted analysis of just the Parkinson's disease cases to investigate the effect of the incidence of dyskinesia, psychosis and autonomic dysfunction on the expression of PAR2, trypsin-2, serpin-A5 and serpin-A13.

Dyskinesia: In the locus coruleus 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 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 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 difference 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 activating serine proteinases trypsin-2 and mast cell tryptase and two serine proteinase inhibitors (serpin-A5 and serpin-A13) in post-mortem brain by immunohistochemistry. There were regional differences in the expression of PAR2 and activating proteinases and proteinase inhibitors in normal brain, which were altered significantly in Parkinson's disease. To present a coherent narrative and in the

absence to any evidence to the contrary, we have taken the small cells counted to be microglia and have termed them as such throughout the manuscript. For stereological counting, a cell was considered positive if staining was darker than the surrounding neuropil. Thus, reduced expression may result in a cell being considered negative because it had similar staining to the neuropil, which 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 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 progressive decrease in neurons positive for proteinase inhibitors (serpin-A5 and serpin-A13) with increasing Braak α -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. In the substantia nigra all proteins examined were significantly decreased and this reflected the profound neuronal loss in this brain region. In contrast, in cingulate cortex, PAR2 had a significant trend to increase with a higher Braak α -synuclein stage rating and serpin-A13 was increased in early Parkinson's disease cases.

With regard to the 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 cingulate cortex) it reflected an increase in the number of microglia that expressed the protein that increased progressively with advancing Parkinson's disease severity (Braak α -synuclein stage).

This study has provided a description of PAR2 expression in Parkinson's disease at different stages of the disease. The work does not purport to be an extensive study of PAR2 expression throughout the brain, but instead provides the first snapshot of the status of PAR2 in Parkinson's disease in various brain regions. Nevertheless, despite this limitation the study does provide novel evidence of altered PAR2 homeostasis and regardless of whether these changes are a part of the disease itself or an epiphenomenon, the detrimental effect of aberrant PAR2 signalling is amenable to drug treatment.

What is the function of PAR2 and proteinases in brain?

In neurons, PAR2 is believed to act as a regulator of proteinase activity through a feedback mechanism that controls release of proteinases to regulate extracellular processes that are

involved in synaptic plasticity (Lohman et al., 2008; Wang et al., 2008). Numerous studies have demonstrated that in hippocampus PAR2 and proteases are involved in synaptogenesis, long-term potentiation and depression and may 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). PAR2 signalling therefore has a neuromodulatory role.

The involvement of PAR2 and serine proteases in long-term potentiation and depression is particularly interesting given the findings of altered trypsin-2 and serpin expression in cases with dyskinesia, since inappropriate long-term potentiation and depression, together with altered dendritic spine density, is thought to underlie treatment-induced dyskinesia in Parkinson's disease (Centonze et al., 2001). Thus, while altered dopamine levels provide the stimulus for synaptic plasticity leading to dyskinesia, it may be that proteinases and PAR2 signalling provide the molecular means by which the dyskinesia manifests. Similarly, while dyskinesia consists of abnormal involuntary movements, L-DOPA-induced psychosis consists of abnormal involuntary thoughts or perceptions (usually visual hallucinations) and dyskinesia and psychosis usually present together in patients (Williams-Gray et al., 2006). 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.

With regard to microglia, PAR2 has been linked with both pro- and anti-inflammatory actions under normal conditions and to pro-inflammatory actions in experimental models of inflammatory brain disease (Afkhami-Goli et al., 2007; Jin et al., 2005; Noorbakhsh et al., 2006). These opposite actions may reflect the dual role that microglia have in the innate immune system. When in surveillance mode ramified microglia constantly sense the environment by sampling of the extracellular space with rapidly and constantly moving processes in order to detect cellular debris or pathogens prior to their removal by the microglia once they are activated and in phagocytotic mode (Kettenmann et al., 2011; Nimmerjahn et al., 2005). Such activity depends on a complex interaction of proteinases and cellular adhesion molecules and occurs in the immediate environment of the cellular process due to low concentrations of the proteinase and tight control of the proteinase by irreversibly acting serpins (Wang et al., 2008). PAR2 therefore plays a part in regulation of microglia activity.

The results of this study, provide evidence that PAR2 homeostasis is disturbed in Parkinson's disease. Consequently, PAR2 signalling could be a factor in the neuroinflammation process that is seen in Parkinson's disease. Testing the effect of a PAR2 antagonist in inflammatory

models of Parkinson's disease, where such drugs would be predicted to be neuroprotective, is therefore required. This view is supported by the recent findings of Liu and colleagues (2014) who demonstrated that inhibition of PAR2 signalling significantly attenuated α -synuclein accumulation and motor dysfunction in a rodent model of Parkinson's disease. In addition, *ex vivo* studies on human periphery macrophages have shown that PAR2 regulates their differentiation, maturation and effector function (Steven et al., 2013) and an antagonist of PAR2 attenuates macrophage activation *in vivo* (Lohman et al., 2012). Microglia are thought to be derived from a macrophage lineage and may therefore be subject to similar regulation via PAR2 in brain.

Serpins

The activity of serine proteinases is tightly regulated by serine proteinase inhibitors (serpins), since uncontrolled proteolysis is clearly undesirable. Serpins comprise a large superfamily of proteins which control processes which require tight regulation such as blood coagulation, inflammation and fibrinolysis (Huntington, 2011). Their role in brain function is unknown, but 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) and now 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 can be increased by tumour necrosis factor (Zhang et al., 2010; Zhang et al., 2012). There were very low numbers of mast cells in the brain regions examined in this study. This was expected, since in humans mast cells are most abundant in young individuals and their density in brain declines with age (Turygin et al., 2005) and the few studies which 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 their physiological role is minor, since the mediators they release can have effects over distances of more than 50 μ 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).

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 which generally becomes worse as the disease progresses. The results at first glance appear contradictory, but PAR biology is complicated and the measured

parameters from these experiments do not necessarily have to interact with each other (Hollenberg et al., 2014). That is, there are likely to be many proteinases in the extracellular milieu that could be activating PAR2 in addition to trypsin-2 that was measured in this study and likewise the trypsin-2 could be acting on other PAR. Similarly, the serpins could be inhibiting a number of proteinases. Hence, the changes measured in this study are not necessary interdependent upon each other. However, if these changes reflect the process of neuroinflammation, then correction of PAR2 activity with drugs might have a beneficial effect on the progression of Parkinson's disease. A view supported by the recent findings of Liu and colleagues (2014), as referred to above. As such, further investigation of the role of PAR2 in Parkinson's disease and in animal models of the disorder is warranted.

Conflict of interest

The authors declare they have no conflicts of interest.

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Figure 1 legend

Representative staining of PAR2, trypsin-2, serpin-A5 and serpin-A13 in each brain region of control brain. Arrows point toward an example of a neuron and arrowheads indicate microglia. Scale bar = 25 μ m.

Figure 2 legend

Representative immunofluorescent staining of PAR2 and TH (tyrosine hydroxylase) in substantia nigra (**A**) and PAR2 and the microglia marker IBA1 (**B**) and the astrocyte marker GFAP (**C**) in cingulate cortex (CgCx) of normal brain. Fluorescent double staining with the PAR2 antibody (green) and IBA1 (microglia) or GFAP (astrocytes) showed that PAR2 was expressed by microglia but not by astrocytes in normal brain. Scale bar = 25 μ m.

Table 1 legend

Demographic details for donors who provided brain tissue. Values are mean $\pm$ standard deviation with range in parentheses for age at death and disease onset, disease duration and post-mortem interval.

Table 2 legend

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, 1990b) and others were bought from reputable companies, except the polyclonal antibody against serpin-A13 which was made to order by Antibody Production Services Ltd. using a synthetic peptide specific to serpin-A13 as the immunogen. Specificity of the antibodies (except serpin-A13) was determined by the supplier by western blot and immunohistochemistry. The anti-serpin-A13 antibody recognized a band of the expected size in western blot which was eliminated when the antibody was preincubated with a 100-fold excess of the immunizing peptide (Supplementary Figure 2). The antibodies for labelling all neurons (neuronal nuclei, neuN), catecholamine neurons (tyrosine hydroxylase, TH), astrocytes (glial fibrillary acidic protein, GFAP), microglia (ionized calcium-binding adapter molecule 1, IBA1) and activated microglia (human leukocyte antigen MHC class II, HLA-DR/DP/DQ) are all established biomarkers and have been used extensively in brain research. Preliminary experiments were conducted to optimize the concentration of antibodies used and determine the best antigen retrieval technique for the available tissue for each antibody.

Table 3 legend

Stereological cell counts of neurons. Immunoreactivity for neurons labelled with the neuronal nuclei marker, PAR2, trypsin-2, serpin-A5, serpin-A13 and tyrosine hydroxylase (catecholamine neuron marker) antibodies was quantified by stereology. Differences between groups (control, incidental Lewy body disease, early and late Parkinson's disease) were

analysed by two-tailed ANOVA with *post hoc* Dunnett multiple comparison test or Kruskal-Wallis non-parametric ANOVA with *post-hoc* pairwise comparison. 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Dunnett's multiple comparison test.

Table 4 legend

Stereological cell counts of microglia. Immunoreactivity for microglia labelled with IBA1, HLA-DR, PAR2, trypsin-2, serpin-A5 and serpin-A13 antibodies were quantified by stereology. Differences between groups (control, incidental Lewy body disease, early and late Parkinson's disease) were analysed by two-tailed ANOVA with *post hoc* Dunnett multiple comparison test or Kruskal-Wallis non-parametric ANOVA with *post-hoc* pairwise comparison. 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Dunnett's multiple comparison test.

Table 5 legend

Summary of significant differences 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 were considered. * $P < 0.05$, ** $P < 0.01$, Dunnett's multiple comparison test.

Figure 1

Figure 2

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

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

^{a, b, c, d}Not significantly different between groups

Table 2 Details of reagents used for immunohistochemistry

Antibody	Supplier	Type	Product number	Antigen retrieval ¹	dilution
<i>Primary</i>					
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
<i>Secondary</i>					
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
<i>Peroxidase conjugate</i>					
Streptavidin-POD	Roche		12213700	-	1:250

¹30 min 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)

Table 3 Stereological cell counts of neurons (cells/mm²)

	Control n = 12	ILBD (Braak 1 or 2) n = 6	Early Parkinson's (Braak 3 or 4) n = 10	Late Parkinson's (Braak 5 or 6) n = 15	correl- ation	ANCOVA
Dorsal motor nucleus						
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	24 ± 4*	*-ve	none
Serpin-A13	113 ± 13	102 ± 12	93 ± 7	77 ± 7	*-ve	none
Tyrosine hydroxylase	31 ± 5	41 ± 6	24 ± 4	28 ± 2	no	none
Locus coeruleus						
Neuronal nuclei	171 ± 12	174 ± 14	150 ± 14	121 ± 12	*-ve	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 (n=9)	62 ± 5**	** -ve	none
Tyrosine hydroxylase	86 ± 12	87 ± 22	57 ± 7	41 ± 6*	** -ve	none
Substantia nigra						
Neuronal nuclei	164 ± 11	163 ± 15	112 ± 16**	97 ± 8***	** -ve	none
PAR2	116 ± 10	94 ± 11	63 ± 9***	59 ± 6***	** -ve	none
Trypsin-2	102 ± 7	88 ± 10	63 ± 7***	59 ± 6***	** -ve	none
Serpin-A5	66 ± 8	64 ± 16	36 ± 11*	37 ± 4*	** -ve	none
Serpin-A13	124 ± 9	119 ± 18	103 ± 26	78 ± 7**	** -ve	none
Tyrosine hydroxylase	117 ± 8	105 ± 19	40 ± 7***	46 ± 3***	** -ve	none
Cingulate cortex						
Neuronal nuclei	283 ± 19	321 ± 28	342 ± 35	315 ± 14	no	none
PAR2	244 ± 12	279 ± 25	251 ± 18	287 ± 11	*+ve	none
Trypsin-2	141 ± 8	145 ± 21	170 ± 16	171 ± 14	no	dyskinesia
Serpin-A5	203 ± 12	202 ± 20	223 ± 21	213 ± 13	no	psychosis
Serpin-A13	328 ± 9	314 ± 13	375 ± 12*	323 ± 12	no	none
Primary motor cortex						
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	dyskinesia
Serpin-A5	198 ± 12	243 ± 21	209 ± 16	209 ± 10	no	dyskinesia
Serpin-A13	308 ± 13	306 ± 18	229 ± 19***	258 ± 11**	** -ve	dyskinesia

Table 4 Stereological cell counts microglia (cells/mm²)

	Control n = 12	ILBD (Braak 1 or 2) n = 6	Early Parkinson's (Braak 3 or 4) n = 10	Late Parkinson's (Braak 5 or 6) n = 15	correl- ation	ANCOVA
Dorsal motor nucleus						
IBA1	212 ± 12	254 ± 24	276 ± 19*	254 ± 18	no	autonomic
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	*+ve	none
Serpin-A5	182 ± 17	243 ± 39	219 ± 24	287 ± 22*	**+ve	none
Serpin-A13	173 ± 15	152 ± 29	135 ± 19	129 ± 12	*-ve	none
Locus coeruleus	n = 13	n = 5	n = 10	n = 15		
IBA1	220 ± 18	353 ± 49*	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	*+ve	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 (n=9)	148 ± 10	no	none
Substantia nigra	n = 13	n = 6	n = 9	n = 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	68 ± 20	35 ± 5	no	dyskinesia
Trypsin-2	54 ± 10	62 ± 19	33 ± 10	42 ± 9	no	none
Serpin-A5	128 ± 13	132 ± 23	95 ± 18	105 ± 10	no	dyskinesia
Serpin-A13	186 ± 19	190 ± 37	233 ± 25	279 ± 14*	**+ve	none
Cingulate cortex	n = 13	n = 6	n = 10	n = 15		
IBA1	304 ± 18	348 ± 28	324 ± 22	316 ± 10	no	none
HLA-DR	94 ± 6	110 ± 16	96 ± 9 (n=9)	91 ± 7	no	none
PAR2	38 ± 6	67 ± 9*	73 ± 11** (n=9)	77 ± 5***	**+ve	none
Trypsin-2	25 ± 2	25 ± 7	38 ± 8	40 ± 6	no	dyskinesia
Serpin-A5	80 ± 11	65 ± 4	67 ± 8	75 ± 5	no	none
Serpin-A13	103 ± 8	91 ± 15	100 ± 8	77 ± 4*	*-ve	none
Primary motor cortex	n = 13	n = 6	n = 10	n = 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	76 ± 18**	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		

Table 5 Summary of significant differences between Parkinson's disease cases when analysed using dyskinesia or psychosis as the independent variable

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

Supplementary Figure 1**A**

Western blot analysis of PAR2 using protein extracted from human primary motor cortex with different post-mortem intervals. A band of the expected size (44 kDa) was detected. Here a gel with three cases from each time group and one from the 48 h group is shown.

B

Unbiased stereological quantification of PAR2 immunoreactivity in human primary motor cortex with differing post-mortem interval. There was no significant difference between any group.

Supplementary Figure 2 **Supplementary Figure 2 legend**

The rabbit anti-serpin-A13 polyclonal antibody raised against a peptide specific for serpin-A13 recognised a band of the expected size (56 kDa) when used in western blot analysis of control primary motor cortex (**A**). Incubation of the antibody with the immunizing peptide obviated the band (**B**). Immunohistochemistry using the anti-serpin-A13 antibody stained neurons and microglia in substantia nigra (**C**, **D**) and cingulate cortex (**E**). When the anti-serpin-A13 was omitted, no staining was observed (**F**). SN, substantia nigra; CgCx, cingulate cortex. Bar = 50 μ m.

Supplementary Table 1 Details of donors who provided brain tissue used in this study

	Age	Gender	Disease onset age	Disease duration	α -synuclein tau stage	tau score	Dyskinesia	Psychosis	Autonomic	PMI hours	Calcification of blood vessels
<i>Control</i>											
	84	M	-	0	0	<2	none	none	none	49	no
	58	M	-	0	0	0	none	none	none	9	no
	82	F	-	0	0	0	none	none	none	27	no
	71	F	-	0	0	0	none	none	none	17	yes
	71	M	-	0	0	0	none	none	none	52	yes
	77	M	-	0	0	<1	none	none	none	48	yes
	81	F	-	0	0	0	none	none	none	15	yes
	93	F	-	0	0	1	none	none	none	22	yes
	74	F	-	0	0	<1	none	none	none	29	no
	65	M	-	0	0	<2	none	none	none	12	no
	78	F	-	0	0	2	none	none	none	23	yes
	80	F	-	0	0	<1	none	none	none	23	yes
	84	F	-	0	0	2	none	none	none	11	yes
	82	M	-	0	0	<1	none	none	none	48	no
<i>Incidental Lewy Body disease</i>											
	104	F	-	0	<2	2	none	none	none	10	yes
	81	F	-	0	<2	2	none	none	none	48	no
	98	F	-	0	<2	6	none	none	none	27	no
	58	M	-	0	<2	0	none	none	none	74	no
	84	F	-	0	<2	<2	none	none	none	?	yes
	98	F	-	0	<2	<2	none	none	none	16	no
<i>Early Parkinson's disease</i>											
	78	M	70	8	3	2	high	low	high	22	yes
	79	M	67	12	3	2	none	high	low	21	no
	76	M	66	10	3	2	none	none	low	10	yes

PAR2 expression in Parkinson's disease

83	M	70	13	4	3	low	low	high	30	no
81	M	67	14	4	2	high	high	high	30	yes
80	F	67	13	4	1	high	high	high	10	no
87	F	77	10	4	2	none	none	low	22	no
72	M	66	6	4	2	high	high	low	9	yes
80	M	72	8	3	2	high	high	high	45	no
86	F	68	18	3	<2	low	high	high	5	no

Late Parkinson's disease

77	M	67	10	6	2	low	low	high	6	no
87	M	74	13	6	2	low	high	low	15	no
80	M	60	20	6	2	high	high	high	16	yes
80	M	75	5	5	<2	low	none	low	7	no
71	F	49	22	6	1	high	high	high	36	no
75	M	65	10	6	2	high	low	high	26	no
78	M	73	5	6	3	low	high	high	5	no
81	F	67	14	6	2	high	high	high	22	yes
75	M	67	8	5	<2	none	none	high	3	yes
61	M	58	3	6	<2	none	none	none	12	yes
83	F	80	3	5	2	low	high	low	12	no
78	M	69	9	5	<2	high	high	none	11	no
80	F	67	13	5	2	high	high	high	24	no
84	F	74	10	5	2	low	high	none	29	yes
73	M	67	6	5	2	low	high	high	5	no
70	F	47	23	5	1	high	high	high	42	no
74	M	72	2	6	<2	none	none	none	10	no
86	M	78	8	5	1	low	high	high	3	yes
82	M	72	10	6	2	none	low	high	10	yes