Alterations of p11 in brain tissue and peripheral blood leukocytes in Parkinson's disease

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Individuals with Parkinson's disease (PD) often suffer from comorbid depression. P11 (S100A10), a member of the S100 family of proteins, is expressed widely throughout the body and is involved in major depressive disorder and antidepressant response. Central p11 levels are reduced in postmortem tissue from depressed individuals; however, p11 has not yet been investigated in PD patients with depression or those without depression. We investigated p11 levels in postmortem PD brains and assessed whether peripheral p11 levels correlate with disease severity. Substantia nigra, putamen, and cortical p11 protein levels were assessed in postmortem brain samples from PD patients and matched controls. In a different set of postmortem brains, p11 mRNA expression was measured in dopaminergic cells from the substantia nigra. Both p11 protein and mRNA levels were decreased in PD patients. Peripheral p11 protein levels were investigated in distinct leukocyte populations from PD patients with depression and those without depression. Monocyte, natural killer (NK) cell, and cytotoxic T-cell p11 levels were positively associated with the severity of PD, and NK cell p11 levels were positively associated with depression scores. Given that inflammation plays a role in both PD and depression, it is intriguing that peripheral p11 levels are altered in immune cells in both conditions. Our data provide insight into the pathological alterations occurring centrally and peripherally in PD. Moreover, if replicated in other cohorts, p11 could be an easily accessible biomarker for monitoring the severity of PD, especially in the context of comorbid depression.

Parkinson's disease | depression | S100A10 | annexin II light chain | biomarker

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide, with a prevalence of 1–2% (1). PD is characterized by a progressive loss of nigrostriatal dopaminergic neurons in the substantia nigra (SN) pars compacta, resulting in bradykinesia, rigidity, and resting tremor (1). Along with the motor symptoms, patients with PD also experience nonmotor symptoms, including depression, hyposmia, sleep disorders, autonomic dysfunction, hallucinations, and cognitive impairment (2). In particular, depression occurs in ∼35% of patients with PD and is often evident at the time of diagnosis (3). The presence of depression is associated with a more rapid decline in motor and cognitive functions in PD patients (4). Striatal dopamine synthesis and dopamine transporter levels are reduced by at least 60% in clinically manifested PD (5). The loss of nigrostriatal dopamine neurons accounts for many of the motor symptoms, which can be symptomatically ameliorated by dopamine receptor agonists, inhibition of dopamine degradation, or replacement with L-DOPA (6). Unfortunately, at present there is no available treatment to slow the progression of PD. Increased insight into the pathophysiology of PD will aid the development of novel treatment modalities.

We have previously demonstrated that L-DOPA potently upregulates p11 in dopaminoceptive neurons, and that p11 is implicated in both beneficial and adverse actions of L-DOPA in rodent models of PD (7–9). p11, a member of the S100 EF-hand protein family, was first identified within a heterotetrameric complex with annexin A2 (10). Previous studies have shown that p11 interacts with G protein-coupled receptors (i.e., serotonin 1B and 4, metabotropic glutamate 5, and CC chemokine 10 receptors) (11–14) and ion channels (including sodium channel Nav1.8, potassium channel subfamily K, acid-sensing ion channels, and transient receptor potential cation channel subfamily V member 5) (15, 16) and increases their levels at the cell surface. Moreover, p11 interacts with enzymes, such as tissue plasminogen activator and phospholipase A2, and, in a tetrameric complex with annexin A2, with the chromatin remodeling factor SMARCA3 (17). Several studies have shown that p11 is highly implicated in depression and in mediating the effects of antidepressant agents (11, 18). Indeed, reduced p11 levels in several brain regions have been reported in depressed individuals and suicide victims (11, 19, 20). Neuronal p11 levels are also reduced in animal models of depression, but are up-regulated by various antidepressant treatments (11, 18). p11 knockout mice exhibit a depression-like phenotype, as well as reduced behavioral improvement and neurogenesis in response to antidepressant regimens (18). We recently developed a flow cytometry-based assay to measure p11 in peripheral leukocytes and have provided preliminary evidence that p11 could serve as a biomarker of antidepressant response using the selective serotonin reuptake inhibitor citalopram (21).

To provide more insight into the pathological mechanisms of PD, in the present study we aimed to quantify p11 mRNA and protein levels in the nigrostriatal system in postmortem brain tissue from PD

Significance

No disease-modifying drugs to slow the progression of Parkinson's disease (PD) are available at present. A deeper understanding of the pathological changes occurring in this disease will suggest novel drug targets and help identify biomarkers for diagnostic and prognostic outcomes. The involvement of p11 in depression and the response to antidepressants is well documented. Up to 35% of patients with PD suffer from comorbid depression. We have found that p11 levels are reduced in PD patients, and, specifically, that peripheral leukocyte p11 levels are altered in PD patients both with depression and without depression in a cell-specific manner. These data provide insight into the pathological alterations occurring centrally and peripherally in PD.

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patients and matched controls. Furthermore, to evaluate a potential biomarker role of p11 in PD, we measured p11 protein in peripheral blood leukocyte populations of carefully phenotyped PD patients with and without comorbid depression.

Results

p11 Levels in Postmortem PD Brains. p11, tyrosine hydroxylase (TH), and actin protein levels were quantified by Western blot analysis in postmortem PD and healthy control (HC) brains. TH was reduced in the nigrostriatal pathway of PD patients compared with HCs, confirming the diagnosis of PD (Fig. 1*A*). p11 levels in the putamen, SN, and cortex were significantly lower in PD patients compared with HCs (two-way ANOVA; $F_{1,24}$ = 27.30, $P < 0.001$, followed by pairwise comparison with a two-sample Student's *t* test; $P < 0.05$, < 0.05, and < 0.01, respectively) (Fig. 1 *A* and *B*). There was also a significant difference between HCs and PD patients when analyzed with the nonparametric Kruskal–Wallis test (H = 16.33). There was no significant difference in actin levels.

P11 mRNA Expression in Dopamine Neurons of the SN Pars Compacta. To confirm the observed reduction in p11 protein levels was not related to the decrease in TH-positive neurons, we quantified p11 mRNA levels in laser capture microdissection (LCM)-isolated dopaminergic cells from PD patients and HCs using real-time quantitative PCR (qPCR). p11 mRNA levels were significantly lower in cells from PD patients compared with HCs ($P < 0.01$, two-sample Student's *t* test) (Fig. 1*C*).

Peripheral p11 Levels Between Groups. We characterized p11 protein levels within monocyte and T-cell subpopulations and NK cells by multicolor flow cytometry. Monocytes were divided into $CD14^+CD16^-$ classically activated monocytes and $CD14^+CD16^+$ non-classically activated monocytes (22). The $CD14^+CD16^+$ cell population also included intermediate monocytes. To semi-quantitatively assess total p11 protein levels, we multiplied the median fluorescence intensity (MFI) of the APC- or PE-conjugated secondary antibody targeting the p11 antibody by the percentage of $p11^+$ cells in each cell population. p11 antibody specificity was confirmed using an isotype control (Fig. 2 *A*–*C*). There was no significant difference between HC and PD patients in the percentage of cells expressing p11 in any cell population examined.

Peripheral blood mononuclear cell (PBMC) p11 levels, in specific cell types, were compared between the HC, PD patients without depression (PD), and PD patients with depression [PD(Dep)] groups. There was no significant difference in p11 levels between males and females in any cell type, and data from both sexes were pooled. p11 protein levels in $CD14^+CD16^-$ cells were significantly higher in PD(Dep) patients compared with HCs ($F_{2,54} = 5.402$, $P = 0.0073$; Tukey's post hoc test, $P < 0.01$) (Fig. 3*A*). There was no significant difference in p11 levels in $CD14^+CD16^+$ cells between these two groups ($F_{2,54}$ = 2.733) (Fig. 3*A*). p11 protein levels in cytotoxic T cells ($CD8^+$) were significantly higher in PD and PD(Dep) patients compared with HCs ($F_{2,45}$ = 12.68, $P = 0.0001$; Tukey's post hoc test, $P < 0.001$) (Fig. 3*B*). There was no significant difference in p11 levels in $CD4^+$, $Foxp3^+$, or NK cells across the three groups ($F_{2,45} = 0.52$, $F_{2,45} = 1.28$, and $F_{2,54}$ = 2.18, respectively) (Fig. 3 *B* and *C*).

Peripheral P11 Levels Within Groups. To characterize PBMC p11 expression within the different patient groups, we compared p11 levels in monocyte and T-cell subtype populations in each group. There was no significant difference in total p11 expression between monocyte subgroups ($CD14^+CD16^-$ vs. $CD14^+CD16^+$) within any patient group (Fig. 3*A*). In contrast, T regulatory cells ($Foxp3^+$) expressed significantly higher levels of p11 than both T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) in each group [$F_{2,42} = 53.53$ for HC, $F_{2,45} = 27.79$ for PD, and $F_{2,48} = 55.13$ for PD(Dep), $P = 0.0001$; Tukey's post hoc test, $P < 0.001$] (Fig. 3*B*). We investigated leukocyte composition to assess whether the observed differences in p11 levels were related to altered

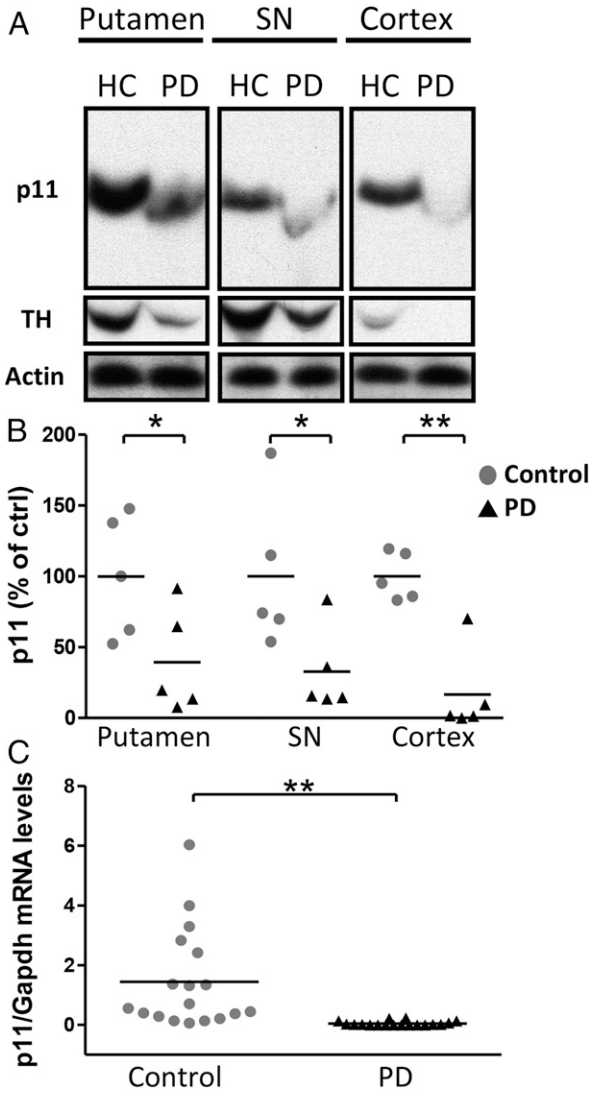

Fig. 1. P11 protein and mRNA levels in postmortem brain tissue from patients with PD and healthy controls. (*A*) Western blots of p11, TH, and actin from HC and PD human postmortem putamen, SN, and cortex. (*B*) Quantification of p11 protein levels from PD patients (n = 5) and HCs (n = 5). p11 levels were normalized to actin. Dots in scatterplots depict individual subjects. (*C*) qPCR analysis of p11 in LCM samples in three biological replicates from six HCs ($n = 3 \times 6 = 18$) and six PD patients ($n = 3 \times 6 = 18$). Scatter diagram showing the mRNA levels of p11 normalized against *Gapdh* levels (n = 18). *$P < 0.05$; **$P < 0.01$ vs. HCs. Data were analyzed using two-way ANOVA and a subsequent pairwise comparison with Student's *t* test (*B*) or using Student's *t* test (*C*).

percentages of total monocytes or T cells, and found no significant difference in PBMC composition among the groups (Fig. S1 *A*–*C*).

P11 Correlates with Clinical Severity in a Cell Type-Specific Manner. To further assess the suitability of p11 as a peripheral biomarker, we correlated p11 levels with patient clinical data. PD disease severity was measured using the unified PD rating scale (UPDRS) and the Hoehn and Yahr (H&Y) scale, and depression scores were measured using the Montgomery–Åsberg Depression Rating Scale (MADRS). p11 levels in $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes were positively associated with total UPDRS score (Fig. 4*A*) and H&Y score (Fig. S2 *A* and *B*) when analyzing all PD patients, both the PD group and the PD(Dep) group [Pearson's correlation, $P < 0.001$ and < 0.01 (Fig. 4*A*) and $P < 0.001$ and < 0.05 (Fig. S2 *A* and *B*), respectively]. Similarly, NK cell p11 levels were positively associated

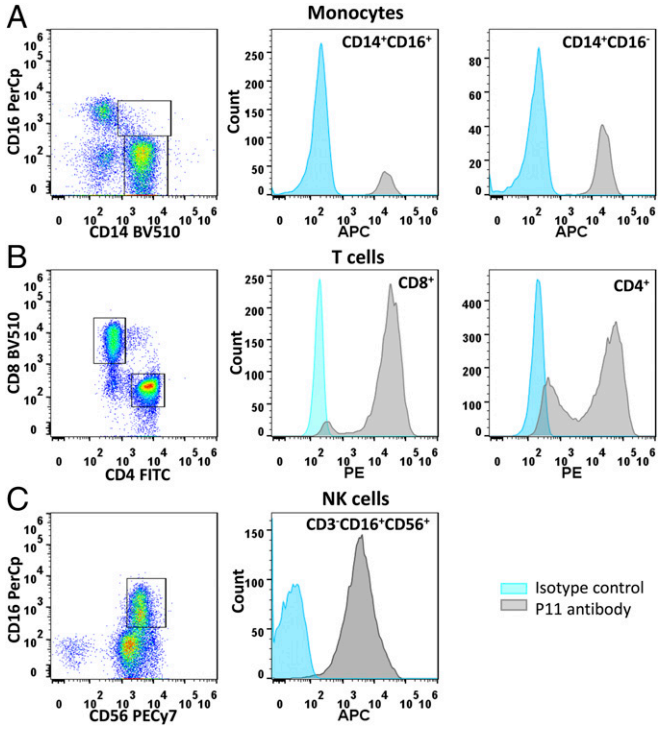

Fig. 2. Specificity of p11 antibody in monocytes, T cells, and NK cells. Representative flow cytometry analysis of PBMCs with primary antibodies toward p11 (gray lines) or IgG1 isotype control (blue lines) in monocytes ($CD14^+CD16^-$ and $CD14^+CD16^+$) (*A*), T cells ($CD8^+$ and $CD4^+$) (*B*), and NK cells ($CD3^-CD16^+CD56^+$) (*C*), demonstrating specific p11 expression.

with total UPDRS and H&Y scores (Pearson's correlation, $P < 0.05$) (Fig. 4*B* and Fig. S2*C*). Cytotoxic T cells ($CD8^+$) were correlated with total UPDRS score (Pearson's correlation, $P < 0.05$) (Fig. 4*C*). When analyzing only the PD(Dep) group, only NK cell p11 levels were significantly associated with MADRS score (Pearson's correlation, $P < 0.01$) (Fig. 4*D*). All correlations were corrected for age, sex, and disease duration. There was no association between p11 level in any T-cell or monocyte population and MADRS score, and p11 levels were not independently correlated with age or disease duration in any cell type investigated.

To assess whether the observed correlations were related to the effects of anti-Parkinsonism medication, we calculated the levodopa daily equivalent dose (LEDD) and analyzed it for a correlation with p11 levels in the distinct cell subtypes. We found no correlation between LEDD score and p11 levels in any cell subtype (Fig. S3 *A*–*F*).

p11 as a Discriminator Between PD Patients and HCs. Given the correlation between peripheral p11 levels and PD severity, we assessed the ability of peripheral p11 levels to discriminate between HCs and PD or PD(Dep) patients using receiver operating characteristic (ROC) curves. Only p11 levels in $CD8^+$ cells could discriminate between HCs and PD patients without depression (Fig. 5*A*), with an area under the ROC curve of 0.97 ($P < 0.0001$). p11 levels in $CD8^+$ cells discriminated between PD patients and HCs with a sensitivity of 93% and specificity of 93% ($\chi^2 = 23.51$, $P < 0.0001$). In contrast, p11 levels in classically activated monocytes ($CD14^+CD16^-$ cells), cytotoxic T cells ($CD8^+$), and NK cells could discriminate between PD(Dep) patients and HCs (Fig. 5*B*), with an area under the ROC curve of 0.76, 0.97, and 0.72, respectively ($P = 0.008$, $P < 0.0001$, and $P = 0.02$, respectively), and a sensitivity of 67% and specificity of 73% ($\chi^2 = 5.6$, $P < 0.05$) in $CD14^+CD16^-$ cells, a sensitivity of 82% and specificity of 93% ($\chi^2 = 18.33$, $P < 0.0001$) in $CD8^+$ cells, and a sensitivity of 67% and specificity of 67% ($\chi^2 = 3.90$, $P < 0.05$) in NK cells.

Discussion

We report here that central and peripheral p11 protein levels are altered in patients with PD, and, furthermore, that p11 protein levels in distinct types of peripheral blood leukocytes are correlated with disease severity and depression scores. Previous studies have found reduced p11 levels in the frontal cortex, nucleus accumbens, and hippocampus in postmortem brain tissue from depressed individuals and suicide victims (11, 19, 20). We demonstrate here that p11 levels in the putamen, SN, and cortex are decreased in postmortem tissue from PD patients. Thus, in PD the reduction of p11 is not limited to the nigrostriatal pathway. Postmortem delay (PMD) may result in experimental artifacts; however, we found no correlation between p11 mRNA levels and PMD, suggesting that the changes observed in p11 mRNA in postmortem tissue are not related to PMD-associated degradation alone. In addition, there was no difference in PMD between HCs and PD patients, and thus any degradation that does occur should occur consistently across the groups. The p11 mRNA variation across biological replicates in the HCs may suggest a p11-enriched subpopulation of dopaminergic neurons. The mechanisms underlying these changes in p11, and the consequences of these changes, are incompletely understood, but the reduction of p11 mRNA in LCM-isolated dopamine neurons indicates a role for transcriptional regulation.

Whether the observed reduction in p11 expression in dopaminergic neurons in PD patients plays a causal role in the disease remains to be studied, and it will be important to study brain tissue from patients with less-advanced PD. Indeed, the current functional role of p11 in dopaminergic neurons is unclear. None of the aforementioned receptors or ion channels known to interact with p11 are abundantly expressed in dopamine neurons. Interestingly, a previous study showed that p11 interacts with

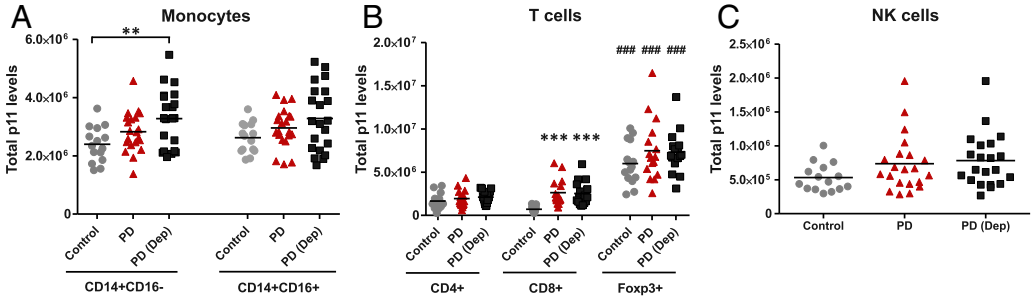

Fig. 3. Flow cytometry analysis of p11 levels in healthy controls and PD patients with and without depression. (*A*) p11 protein levels in classically activated ($CD14^+CD16^-$) and non-classically activated ($CD14^+CD16^+$) monocytes from HCs, PD patients, and PD(Dep) patients ($n = 15$, 21, and 21, respectively). (*B*) p11 protein levels in T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and T regulatory cells ($Foxp3^+$) from HCs and PD and PD(Dep) patients ($n = 15$, 16, and 17, respectively). (*C*) p11 protein levels in NK cells ($CD3^-CD16^+CD56^+$) from HCs and PD and PD(Dep) patients ($n = 15$, 21, and 21, respectively). Data are expressed as total p11 levels (MFI × % $p11^+$ cells). In *A*–*C*, dots in the scatterplots depict individual subjects. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the same cell type in HCs; $^{\#\#\#}P < 0.001$ vs. $CD4^+$ and $CD8^+$ cells in the same participant group. Data were analyzed using one-way ANOVA and Tukey's post hoc test.

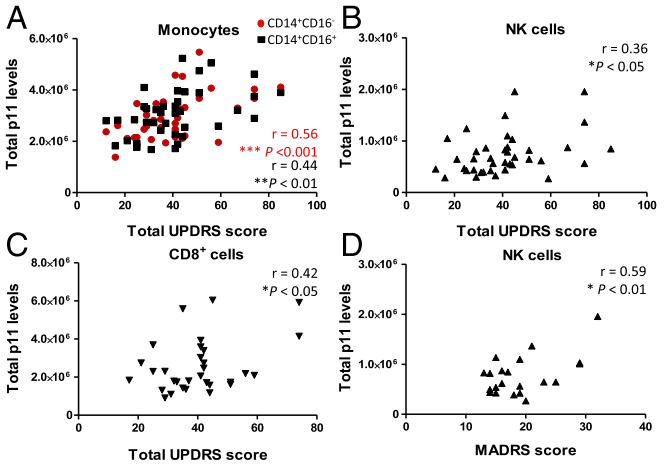

Fig. 4. Association of peripheral p11 levels and clinical characteristics in distinct cell types. (*A–C*) Graphs showing positive correlations between total p11 levels in classically activated monocytes ($CD14^+CD16^-$; $n = 40$) and non-classically activated monocytes ($CD14^+CD16^+$; $n = 39$) (*A*), NK cells ($CD3^-CD16^+CD56^+$; $n = 40$) (*B*), and cytotoxic T cells ($CD8^+$; $n = 32$) (*C*) from all PD patients, with PD disease severity assessed by UPDRS score. (*D*) Graph showing a positive correlation between total p11 levels in NK cells from PD (Dep) patients and depressive symptoms, as measured by MADRS score ($n = 21$). Dots represent individual patients. Data were analyzed using Pearson's correlation test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Bcl-xL/Bcl-2–associated death promoter (BAD) and dampens its proapoptotic activity (23), suggesting that reduced p11 may contribute to cell death. It will be important to further elucidate the role of p11 in dopamine neurons, particularly in relation to mechanism(s) underlying neurodegeneration.

The diagnosis of PD is based solely on clinical assessment (24), which often leads to misdiagnosis (25). Reproducible and robust objective biomarkers to support the diagnosis, classify subtypes, and track disease progression would represent a scientific breakthrough (26). Given our finding of altered p11 levels in several brain regions of PD patients, the need for biomarkers, and the correlation between changes in p11 levels and responses to antidepressant agents, we investigated peripheral levels of p11 in PBMCs of PD patients with and without depression. We found that leukocyte p11 levels are altered in PD patients in a cell type-specific manner. Taking into account the differential yet linked functions of the various cell subtypes present in PBMCs, we considered it of interest to measure p11 levels in specific cell subsets. Furthermore, p11 protein expression is almost 10-fold higher in monocytes than in other leukocytes (21), and thus, measuring p11 protein levels in whole blood by, e.g., Western blot analysis may mask cell-specific differences. To capture any discrete changes in blood p11 levels, we measured p11 levels in classically activated monocytes, nonclassically activated monocytes, NK cells, and a variety of T-cell subsets. We extend previously reported data by demonstrating higher p11 protein levels in T regulatory cells compared with both cytotoxic and T helper cells under basal and pathological conditions. T regulatory cells are involved in dampening the immune system, and a role for them is also emerging in depression (27, 28); thus, the higher p11 levels in these cells are of interest.

p11 levels are increased in classically activated ($CD14^+CD16^-$) monocytes from PD patients with depression, but not in PD patients without depression, suggesting that this change may be due to the depressive symptoms rather than to PD per se. In this study, it was not possible to examine an age- and sex-matched group of patients with major depression. In a previous study, we assessed p11 levels in all monocytes from patients with major depression and matched controls and did not find any significant differences (21). Future work will aim to assess whether p11 levels are increased in classically activated monocytes from depressed patients without PD. Both patient groups had increased p11 levels in cytotoxic T cells, suggesting that this effect may be related to PD rather than to depression. Given that these changes were not present in non-classically activated monocytes or in other T-cell subsets, it is plausible that p11 is differentially regulated in various PBMC cell types under pathological conditions, and, moreover, that p11 may be alternatively regulated within the adaptive and innate immune system. The patients examined here had PD of varying duration; it will be of interest to examine de novo PD patients to investigate whether these alterations in p11 are present at early stages of the disease.

We have previously provided evidence that p11 levels in monocytes and NK cells are correlated with treatment response in major depressive disorder (21). We thus assessed whether p11 levels are correlated with patients' clinical characteristics. Specifically, we investigated PD severity as measured by the UPDRS and depression score as measured by the MADRS. The UPDRS takes into account limitations of daily life, motor and nonmotor symptoms, and complications of therapy. A weakness of this study is that patients were evaluated during the on-phase, and as such, correlation of p11 levels with UPDRS III was not appropriate. However, patients were also scored on the H&Y scale, which only accounts for motor dysfunction, and the correlations remained significant for both monocytes and NK cells with this rating scale. p11 levels in monocyte subsets were positively associated with total UPDRS score. Similarly, p11 levels in cytotoxic T cells and NK cells were also correlated with disease severity. These correlations were independent of PD treatment; we found no significant correlations between LEDD score and p11 levels in any cell type. All PD patients were included in these analyses. When the PD and PD(Dep) subgroups were investigated separately, the correlations did not remain significant, owing to the relatively low number of patients in each group. In line with our previous report (21), p11 levels in NK cells were correlated with the MADRS depression score in PD(Dep) patients. Zorrilla et al. (29) reported a decreased number of NK cells in depressed patients; moreover, Grosse et al. (30) reported that NK cell activity is related to antidepressant response. Our data add to the emerging evidence supporting an association between NK cells and a depressive phenotype, even with a comorbidity of PD.

A previous study has shown an increase in infiltrating peripheral cells in the SN of PD patients (31), and PBMC composition and function are reportedly altered in PD patients (32–36). To ensure that our observed differences in p11 levels were not related to PBMC composition changes, we also quantified leukocyte composition. We did not observe any alteration in the percentages of monocytes or T-cell subpopulations in either PD group or the HCs in our cohort.

p11 levels in monocytes, cytotoxic T cells, and NK cells were all correlated with PD severity. Interestingly, only p11 levels in $CD8^+$ cells were able to discriminate both PD and PD(Dep) patients from HCs, with similar levels of specificity and sensitivity. This is

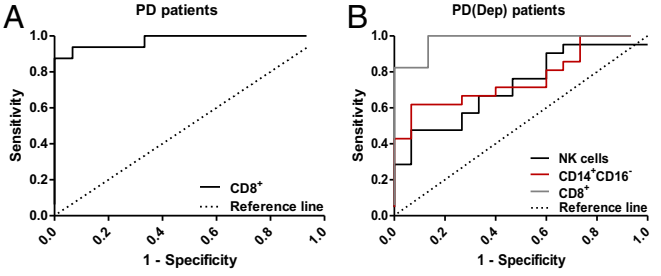

Fig. 5. ROC curve of peripheral p11 levels as a discriminant function between PD patients and HCs. (*A*) $CD8^+$ p11 levels as a discriminant function between PD patients without depression and HCs. $^{***}P < 0.001$. (*B*) NK cell, $CD14^+CD16^-$ cell, and $CD8^+$ cell p11 levels as a discriminant function between PD(Dep) patients and HCs. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in line with the fact that only $CD8^+$ p11 levels were altered in both PD groups. p11 levels in classically activated monocytes and NK cells could only discriminate PD(Dep) patients from HCs, and with lower sensitivity and specificity than p11 levels in $CD8^+$ cells. This could indicate a stronger relationship between monocytes and NK cells with depression, whereas cytotoxic T-cell p11 levels may be more affected by PD, as mentioned previously. None of the aforementioned cell types investigated could discriminate between the two PD groups.

We previously found that p11 levels are reduced in the cingulate cortex and the nucleus accumbens in depressed patients (11, 20), and now report that p11 is reduced in the putamen, SN, and cortex in PD patients. Another similarity between these conditions is that in some classes of leukocytes, peripheral levels of p11 are regulated in an opposing direction compared with regulation in the CNS. In PD, reduced p11 levels are found in nigrostriatal neurons, whereas elevated p11 levels are found in monocytes and cytotoxic T cells. Future work is needed to clarify the mechanism(s) regulating p11 expression in different cell types. In this context, it should be noted that bidirectional regulation of a protein centrally vs. peripherally does not preclude its potential as a clinically useful biomarker. For instance, even though peripheral levels of β-amyloid in cerebrospinal fluid are reduced, β-amyloid remains the most robust peripheral biomarker to aid the diagnosis of Alzheimer's disease (37).

In conclusion, this study provides further evidence that p11 levels are altered in common psychiatric and neurologic disorders. We found decreased p11 levels in several brain regions in patients with PD. The possible role of altered p11 levels in the etiology of PD, however, remains to be identified. In particular, it will be important to elucidate the possible role(s) of p11 in the neurodegeneration of dopamine neurons. The fact that p11 is differentially regulated in leukocytes and is positively correlated with disease severity and depression symptomatology in specific cell populations suggests its possible use in the development of biomarkers for PD, if replicated in independent PD cohorts.

Materials and Methods

Participants. All human experiments were carried out in accordance with the Declaration of Helsinki. The patient investigations were approved by the Ethical Committee of the each participating institution. Each subject provided informed consent at his or her respective institution.

Postmortem Human Brain Tissue. All postmortem tissue specimens were age- and sex-matched. For protein analysis, fresh snap-frozen postmortem brain tissue from PD patients ($n = 5$; 3 females and 2 males) and HCs ($n = 5$; 4 females and 1 male) was obtained from the Debrecen University brain bank. PD patients were defined based on standard criteria, and the inclusion criteria for control subjects was the absence of PD. The mean age of HCs and PD patients was not significantly different (84 ± 9.4 y vs. 82.8 ± 5.8 y). All subjects had a PMD of <10 h. For LCM, fresh snap-frozen human brain tissue was obtained from the Banner Sun Health Research Institute brain bank. Donors were recruited as described previously (38). The mean age of HCs ($n = 6$; 1 female and 5 males) and PD patients ($n = 6$; 2 females and 4 males) was not significantly different (81.5 ± 14.7 y vs. 81.8 ± 7.9 y). The average PMD also was not significantly different between the two groups (HCs, 3.19 ± 0.97 h; PD patients, 2.88 ± 0.79 h).

Participants for PBMC Isolation. Participants for measurement of peripheral p11 protein levels were recruited from the Neurology Clinic at the Karolinska University Hospital, Stockholm, Sweden and gave informed consent. Patients were scored using the UPDRS, the H&Y scale, and the MADRS. A total of 42 patients with PD were included in the study; demographic and clinical characteristics are presented in Table 1. PD patients with a MADRS score >13 were considered to exhibit symptoms of depression and were included in the PD(Dep) group ($n = 21$), whereas those with a MADRS score of ≤5 were considered to not have depression ($n = 21$) and were included in the PD group (Table 1). The subjects included in this study represent a cohort of patients at different disease stages, ranging from de novo PD patients to those with a H&Y score of 4 (Table 1). Patient LEDD scores were calculated as described by Tomlinson et al. (39) on the day before blood draws. Inclusion criteria for HCs ($n = 15$) were the absence of PD, absence of depression, and absence of any disorder affecting the immune system. Groups were age- and sex-matched. It was not possible to examine an age- and sex-matched group of patients with major depression in this study.

Western Blot Analysis. The putamen, SN, and cortex (pooled tissue from temporal, prefrontal, and parietal cortex) were microdissected out from fresh-frozen postmortem coronal sections from five HCs and five PD patients. Frozen tissue was sonicated in 1% SDS and boiled. Protein concentration was determined using the Pierce bicinchoninic acid protein assay method (Thermo Fisher Scientific). Equal amounts of protein were separated by SDS/PAGE and transferred to Immobilon-P (PVDF) membranes. Immunoblotting for p11 was carried out with a monoclonal mouse anti-human p11 antibody (1/1,000, clone 148, annexin II light chain; BD Biosciences) (11) and for TH and actin using polyclonal rabbit antisera (1/1,000; Millipore and 1/1,000; Sigma-Aldrich, respectively). Immunoreactive bands were detected by enhanced chemiluminescence and quantified by densitometry, using National Institutes of Health ImageJ 1.63 software. The level of p11 was normalized to the level of actin. All data are presented as normalized values.

LCM and qPCR. p11 mRNA in dopaminergic cells from postmortem human brain tissue from six PD patients and six HCs was measured by qPCR. Cryosectioning was used to obtain 10-μm-thick sections of the ventral midbrain. Sections were mounted on membrane glass slides (Zeiss) at −20 °C. Before LCM, the tissue was dehydrated by a series of ethanol washes. For each sample, 100 neuromelanin-positive neurons were captured using a Leica LCM system. The Smart-Seq2 protocol was used to produce the cDNA (40). cDNA integrity was assessed using an Agilent 2100 bioanalyzer, and real-time qPCR was performed using Taqman Gene Expression Assay probes (Hs02758991_g1 for glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and Hs00751478_s1 for *p11*; Applied Biosystems). p11 expression values were normalized to Gapdh. Data were analyzed using a two-sample Student's *t* test and are presented as mean ± SD. Three biological replicates (each consisting of 100 cells) were used for each patient or control, for a total of 18 samples analyzed for each group.

Blood Collection and Leukocyte Preparation. Using a standard procedure, blood samples were collected by venipuncture using tubes containing EDTA at the Karolinska University Hospital and processed within 4 h. PBMC cell suspensions were prepared by low-density gradient centrifugation using Lymphoprep (Axis-Shield) and then frozen at −80 °C in freezing medium containing 10% dimethyl sulfoxide and 90% FBS.

Flow Cytometry. Frozen PBMCs were thawed quickly and washed with PBS. Cells were stained with a near-IR dead cell marker (Invitrogen) before being fixed and permeabilized for subsequent intracellular p11 staining using a monoclonal mouse anti-human p11 antibody (2.5 μg/mL, clone 148; BD Biosciences) or an isotype control mouse IgG monoclonal antibody (2.5 μg/mL, clone MOPC-21;

Table 1. Demographic and clinical characteristics of study participants

Group	Age, y, mean ± SD	Sex, females/ males, n	Disease duration, y, mean ± SD	H&Y score, median ± SD	Total UPDRS, mean ± SD	MADRS score, mean ± SD	MoCa, mean ± SD	LEDD, mean ± SD	SSRI, *n*	SNRI, *n*	AAP, *n*	BZD, *n*	AID, *n*
PD	69.1 ± 5.5	4/17	7.6 ± 6.5	2 ± 0.7	32.4 ± 9.7	1.7 ± 1.5	24.9 ± 2.9	831 ± 596	0	3	1	2	3
PD(Dep)	69.1 ± 6.8	4/17	7.5 ± 5.6	2 ± 1	50.8 ± 18	19.2 ± 5.5	23.6 ± 5.7	720 ± 551	4	5	3	6	0
HC	68.1 ± 8.2	7/9											1

AAP, atypical antipsychotic; AID, anti-inflammatory drug; BZD, benzodiazepine; MoCa, Montreal Cognitive Assessment; SNRI, serotonin and noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.

Nordic BioSite) (Fig. 2 *A–C*), and thereafter with an APC-conjugated (to identify p11 in monocyte and NK cells; 1-40; Dako) or PE-conjugated (to identify p11 in T cells; 1–100; Nordic BioSite) rabbit anti-mouse antibody. Cells were subsequently washed and incubated in blocking buffer (1% mouse serum and 1% FBS in permeabilization buffer). Cells were incubated in a mixture of antibodies to identify classically activated monocytes ($CD14^+CD16^-$), non-classically activated monocytes ($CD14^+CD16^+$), and NK cells ($CD3^-CD16^+CD56^+$)—CD3FITC clone UCHT1, CD16PerCp clone 3G8, CD56PeCy7 clone B159, and CD14BV510 clone M5E2—or cytotoxic T cells ($CD8^+$), T helper cells ($CD4^+$), and T regulatory cells ($Foxp3^+$)—CD4FITC clone OKT4, CD3PerCP/Cy5.5 clone UCHT1, and CD8BV51 clone SK1.

For regulatory T-cell identification, cells were fixed and permeabilized a second time, and then incubated in either an AF647-conjugated mouse anti-human Foxp3 antibody (1.5 μg/mL; clone 206D) or an AF647 conjugated mouse anti-human isotype control antibody (1.5 μg/mL; clone MOPC-21). Stained cells were analyzed by multicolor flow cytometry using a Beckman Coulter Gallios flow cytometer. Data were analyzed with Flow Jo (Tree Star) using an appropriate color compensation matrix, and isotype control antibodies were used to confirm antibody specificity for p11 (Fig. 2 *A–C*). A hierarchical gating strategy was used to remove doublets and dead cells, identify cell subtypes, and subsequently quantify p11 staining within each specific cell type (Figs. S4–S6). Monocyte and NK cell p11 levels were measured in 21 PD patients and 21 PD(Dep) patients, and T-cell p11 levels were measured in 16 PD patients and 17 PD(Dep) patients, owing to the greater number of cells required for the T-cell analysis.

Statistical Analysis. Statistical analysis was carried out using GraphPad Prism 5. One-way ANOVA with Tukey's post hoc test, two-way ANOVA, the Kruskal–Wallis test, two-sample Student's unpaired *t* test, or Pearson's correlation was used as appropriate, and all correlations were corrected for age, sex, and disease duration. Normality of distribution was evaluated using the D'Agostino and Pearson omnibus normality test. Statistical significance was set at $P < 0.05$. ROC analyses were conducted to obtain the area under the ROC curve. Cutoff scores were based on the highest sensitivity and specificity combinations for χ^2 analysis.

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