ANNALS of Clinical and Translational Neurology
Open Access
ANA American Neurological Association since 1875

RESEARCH ARTICLE

Dysregulation of protein phosphatase 2A in parkinson disease and dementia with lewy bodies

Hye-Jin Park[1,†], Kang-Woo Lee[1,†,a], Eun S. Park[1,b], Stephanie Oh[1], Run Yan[1], Jie Zhang[1], Thomas G. Beach[2], Charles H. Adler[3], Michael Voronkov[4], Steven P. Braithwaite[4,c], Jeffry B. Stock[4,5] & M. Maral Mouradian[1]

[1]Center for Neurodegenerative and Neuroimmunologic Diseases, Department of Neurology, Rutgers – Robert Wood Johnson Medical School, Piscataway, New Jersey 08854
[2]Banner Sun Health Research Institute, Sun City, Arizona 85372
[3]Mayo Clinic, Scottsdale, Arizona 85259
[4]Signum Biosciences, 133 Wall Street, Princeton, New Jersey 08540
[5]Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

Correspondence
M. Maral Mouradian, Department of Neurology, Rutgers - RWJMS, 683 Hoes Lane West, Room 180, Piscataway, NJ, 08854. Tel: 732-235-4772; Fax: 732-235-4773; E-mail: m.mouradian@rutgers.edu

Current address
[a]Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Republic of Korea
[b]Albert Einstein College of Medicine, Bronx, New Jersey 10461
[c]Alkahest, 75 Shoreway Drive, Suite D, San Carlos, California 94070

Funding Information
This study was supported by a grant from the Michael J. Fox Foundation for Parkinson's Research to S.P.B. and M.M.M. T.G.B. is supported by grants from the NIA, NINDS, Michael J. Fox Foundation and the State of Arizona. J.B.S. is supported by NIH grant AT006868. M.M.M. is the William Dow Lovett Professor of Neurology and is supported by the American Parkinson Disease Association and by NIH grants AT006868 and NS073994. The Banner Sun Health Research Institute Brain and Body Donation Program is supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson's Disease and Related Disorders), the National Institute on Aging (P30 AG19610 Arizona Alzheimer's Disease Core Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05-901 and 1001 to the

Abstract

Objective: Protein phosphatase 2A (PP2A) is a heterotrimeric holoenzyme composed of a catalytic C subunit, a structural A subunit, and one of several regulatory B subunits that confer substrate specificity. The assembly and activity of PP2A are regulated by reversible methylation of the C subunit. α-Synuclein, which aggregates in Parkinson disease (PD) and dementia with Lewy bodies (DLB), is phosphorylated at Ser_{129}, and PP2A containing a B55α subunit is a major phospho-Ser_{129} phosphatase. The objective of this study was to investigate PP2A in α-synucleinopathies. **Methods**: We compared the state of PP2A methylation, as well as the expression of its methylating enzyme, leucine carboxyl methyltransferase (LCMT-1), and demethylating enzyme, protein phosphatase methylesterase (PME-1), in postmortem brains from PD and DLB cases as well as age-matched Controls. Immunohistochemical studies and quantitative image analysis were employed. **Results**: LCMT-1 was significantly reduced in the substantia nigra (SN) and frontal cortex in both PD and DLB. PME-1, on the other hand, was elevated in the PD SN. In concert with these changes, the ratio of methylated PP2A to demethylated PP2A was markedly decreased in PD and DLB brains in both SN and frontal cortex. No changes in total PP2A or total B55α subunit were detected. **Interpretation**: These findings support the hypothesis that PP2A dysregulation in α-synucleinopathies may contribute to the accumulation of hyperphosphorylated α-synuclein and to the disease process, raising the possibility that pharmacological means to enhance PP2A phosphatase activity may be a useful disease-modifying therapeutic approach.

Arizona Parkinson's Disease Consortium), and the Michael J. Fox Foundation for Parkinson's Research.

Received: 20 May 2016; Accepted: 8 July 2016

Annals of Clinical and Translational Neurology **2016; 3(10): 769–780**

doi: 10.1002/acn3.337

[†]HJP and KWL contributed equally to this work

Introduction

Protein phosphorylation is a tightly regulated posttranslational modification that impacts nearly all cellular processes. A balance is normally maintained through the actions of multiple kinases and phosphatases. Protein phosphatase 2A (PP2A) is a ubiquitously expressed conserved enzyme, with its catalytic C subunit constituting up to 1% of total cellular proteins, and accounts for the majority of serine/threonine phosphatase activity in the brain.[1,2] In neuronal cells, most of the C subunit is associated with a conserved scaffold-like A subunit, and one of several different regulatory B subunits that confer different substrate specificities on the resulting trimeric holoenzymes. The binding of different B subunits to the AC dimer is regulated by reversible carboxyl methylation of the C subunit.[3–5] Thus, PP2A methylation is critical for its selective phosphatase activity toward different phospho-protein substrates. The methylation status of PP2A is governed by the opposing activities of a PP2A-specific leucine carboxyl methyltransferase (LCMT-1) and a PP2A-specific methylesterase (PME-1).[6–8]

α-Synuclein is an abundant protein in the brain that misfolds and polymerizes into fibrillar form in Lewy bodies and Lewy neurites, which are pathologic hallmarks of α-synucleinopathies such as Parkinson disease (PD) and dementia with Lewy bodies (DLB).[9] α-Synuclein is extensively phosphorylated at Ser_{129} in PD and DLB brains.[10–12] Additionally, hyperphosphorylated and misfolded α-synuclein accumulates in neurons of transgenic mice that express human α-synuclein,[11,13] and hyperphosphorylation exacerbates α-synuclein toxicity in *Drosophila*.[14] In vitro, phosphorylation of α-synuclein at Ser_{129} promotes its oligomerization and fibrillization.[10]

We demonstrated previously in in vitro experiments and in cultured neuroblastoma cells that the main enzyme that dephosphorylates α-synuclein is the B55α containing isoform of PP2A.[13] We also identified a naturally occurring serotonin derivative in coffee, eicosanoyl-5-hydroxytryptamide (EHT), that inhibits PME-1-dependent PP2A demethylation, thereby stabilizing the PP2A heterotrimeric holoenzyme AB55αC that dephosphorylates α-synuclein. α-Synuclein transgenic mice maintained on a diet supplemented with EHT exhibited dramatically reduced α-synuclein phosphorylation and aggregation in the brain, accompanied with enhanced neuronal integrity and reduced neuroinflammation as well as improved motor performance.[13]

Based on these preclinical observations, we hypothesized that alterations in PP2A methylation may contribute to the pathology of human α-synucleinopathies.,[15] particularly in light of decreased PP2A activity reported in the brains of individuals with DLB and α-synuclein triplication.[16] Abnormal regulation of PP2A has been noted in another neurodegenerative disorder, Alzheimer's disease (AD), with decreased phosphatase activity and reduced PP2A methylation in postmortem brains.[17,18] Furthermore, PP2A has been shown to be the major phosphatase acting on phospho-tau,[15] and EHT ameliorates the neurodegenerative phenotype in a rat model of AD.[19]

This study examines the state of PP2A methylation in postmortem brains of patients with PD and DLB as well as the expression of the two enzymes that control PP2A methylation, LCMT-1, and PME-1. The results show robust abnormalities compared to control brains consistent with compromised PP2A activity that can contribute to an abnormally elevated phosphorylation state of α-synuclein in these disorders.

Materials and Methods

Brain samples

Human postmortem brain tissue sections that were fully characterized clinically and neuropathologically were obtained from the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona.[20] Deidentified samples from eight subjects in each

of the diagnostic groups consisting of Parkinson disease (PD), dementia with Lewy bodies (DLB), and non-neurological controls (Controls) were studied (Table 1). The three groups were age-matched with mean age at death of 79 ± 2.8 years for PD cases, 82 ± 2.6 years for DLB, and 84 ± 2.1 years for Controls (not significant). Gender distribution was equal for both PD and DLB cases, while the Control group included five males and three females. Only one PD case had a positive family history of tremor. Family history among DLB cases included two AD, two others dementia, and one tremor. All PD cases were tested for glucocerebrosidase (GBA) and LRRK2 mutations; three were found to have GBA mutations, and one had LRRK2 mutation. None of these GBA or LRRK2 mutants had positive family history. Postmortem interval (PMI) was ≤3 h in all but one PD subject. United Parkinson Disease Rating Scale (UPDRS) motor scores in the "practically-defined off state" in all but three subjects were 39.4 ± 8.5 for PD, 35.7 ± 11.4 for DLB, and 10.4 ± 4.3 for Controls ($P < 0.05$ for difference between PD and Controls). Mean Mini-Mental State Examination scores were 18 ± 3.6 in the PD group, 8.8 ± 3.1 in DLB and 27.1 ± 0.8 in Controls ($P < 0.01$ for difference between DLB and Controls). Lewy bodies were detected in PD and DLB brains but in none of the Controls. Neuropathological stage of α-synucleinopathy assigned according to the Unified Staging System for Lewy Body Disorders [21] showed all DLB cases to be in stage 4, PD cases ranged from stages 2 to 4, and all the Controls were in stage zero. All PD-affected brains had severe depigmentation of the substantia nigra, while those with DLB had mild to moderate depigmentation, and all but one Control had no or mild depigmentation.[22] Concomitant Alzheimer pathology was also present. Mean neuritic plaque density determined according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [23] was 1.0 ± 0.3 for PD, 2.8 ± 0.2 for DLB, and 1.0 ± 0 for Controls ($P = 0.001$ for difference between DLB and Controls, and $P < 0.01$ for PD vs. DLB), and neurofibrillary tangles assessed using Braak staging [24] showed mean scores of 2.9 ± 0.4 for PD, 4.5 ± 0.3 for DLB, and 2.8 ± 0.2 for Controls ($P = 0.01$ for difference between DLB and Controls, and $P < 0.05$ for PD vs. DLB). Demographic and neuropathological details are provided in Table 1.

Immunohistochemistry

For immunohistochemical analysis, 40 μm free-floating formalin-fixed sections of the substantia nigra and middle frontal gyrus of the cerebral cortex stored at 4°C were used. After washing with phosphate-buffered saline (PBS), tissue sections were incubated in 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity. To enhance epitope antigenicity, samples were incubated in preheated sodium citrate (pH 6.0) for 30 min at 75°C. Sections were then blocked using 5% BSA for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: antibody for LCMT-1 (also known as PPMT) (1:100, a kind gift from Egon Ogris)[17]; 07-095 for PME-1 (1:500, Millipore); 6A3 for methylated-PP2A (1:200, generated at Princeton University)[4]; 1D6 for demethylated-PP2A (1:1000; Millipore)[25]; 06-222 for total PP2A C subunit (1:500, Millipore); and 2G9 for total B55α subunit of PP2A (1:500, Millipore). Sections were washed with PBS-Tween (PBS-T) three times and incubated with biotinylated secondary antibody for 1 h. After washing again with PBS-T, sections were incubated with biotinylated HRP complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, followed by incubation with 3,3′-diaminobenzidine (DAB) for color development. Hematoxylin-stained adjacent sections of the substantia nigra (SN) were used to count neuromelanin-containing neurons. All samples across different diagnostic groups were stained under the same conditions. For image analysis, tissue sections were observed under a Nikon Eclipse 55i light microscope, images were captured using NIS-Elements D software (Nikon), and the area occupied by immunoreaction in each image was quantified using the thresholding tool in ImageJ (NIH, Bethesda, MD). The mean areas for each disease group were then compared to that of the Control group. Signals in the SN were normalized against the density of residual neuromelan-containing neurons.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analysis and graphic representation. Data are presented as means ± SEM. Differences among the three groups in the levels of LCMT-1, PME-1, methylated PP2A, demethylated PP2A, total PP2A, and B55α were analyzed using one-way analysis of variance (ANOVA) with post hoc Bonferroni's multiple comparison test. Differences in clinical and demographic characteristics (age, Mini-Mental State Examination, postmortem interval, motor UPDRS, Braak stage, and neuritic plaque density) were analyzed using Kruskal–Wallis ANOVA with post hoc Dunn's multiple comparison test. $P < 0.05$ were considered statistically significant.

Results

A marked decrease in the area occupied by immunoreaction for the PP2A methylating enzyme LCMT-1 was found in the SN of both PD and DLB brains, and a lesser,

Table 1. Postmortem brain tissue samples, and the clinical and neuropathologic profile of study subjects.

Group	Gender	Age at Death	PMI	Motor UPDRS	MMSE Score	Unified LB Stage	SN depigmentation	Plaque density	Braak score
Non-neurological Controls									
1	Male	81	2.75	6.5	25	0	None	1	III
2	Female	90	3	13	27	0	Moderate	1	III
3	Male	85	1.83	6	30	0	None	1	II
4	Male	82	3	0	26	0	Mild	1	III
5	Male	82	2.16		28	0	Mild	1	III
6	Male	73	2.5	4	30	0	Mild	1	II
7	Female	88	2	34.5	24	0	None	1	III
8	Female	91	2.5	9	27	0	None	1	III
Parkinson's Disease									
1	Female	73	2.16	78	19	4	Severe	1	III
2	Male	70	1.83	30.5[1]	8	3	Severe	0	III
3	Male	85	2	12[1]	28	3	Severe	2	IV
4	Female	82	3	38[1]	16	3	Severe	1	III
5	Male	78	2.5	37	19	2	Severe	0	III
6	Male	69	2.25	74	0	4	Severe	1	II
7	Female	87	2.16	24	28	3	Severe	1	I
8	Female	89	3.66	22	27	2		2	IV
Dementia with Lewy Bodies									
1	Female	76	3	10	4	4	Moderate	2	IV
2	Female	80	2.33		0	4	Moderate	2	III
3	Male	69	2.5	79	0	4	Moderate	3	V
4	Male	86	2.83	47	10	4	Moderate	3	IV
5	Male	88	2.16	8	26	4	Mild	3	IV
6	Female	82	2.66	72	5	4	Moderate	3	V
7	Female	92	2.5	12	15	4	Mild	3	VI
8	Male	86	2.3	22	10	4	Moderate	3	V

PMI, postmortem interval; UPDRS, United Parkinson Disease Rating Scale, Motor subscale; MMSE, Mini-Mental State Examination; Unified LB Stage, Unified Staging System for Lewy Body Disorders: 0 = stage 0 (no Lewy bodies); 2 = Stage IIa (Brainstem predominant) and Stage IIb (Limbic predominant); 3 = Stage III (Brainstem and Limbic); and 4 = Stage IV (Neocortical). SN = substantia nigra pars compacta. CERAD neuritic Plaque Density scores are 0 = none; 1 = sparse; 2 = moderate; 3 = frequent. Braak score staging is for neurofibrillary tangles.
[1]These subjects were evaluated with the Motor UPDRS in the "ON" state; all other subjects were evaluated off antiparkinson drugs.

but still significant, decrease was detected in the cortex of both diseases in comparison to Controls (Fig. 1). In SN, LCMT-1 immunoreaction was decreased by 63% in PD ($P < 0.0001$) and 55% in DLB ($P < 0.0001$), while in the cortex, this was reduced by 30% in PD ($P < 0.01$) and by 28% in DLB ($P < 0.01$) compared to Controls. Conversely, the demethylating enzyme PME-1 was elevated in the SN in PD ($P < 0.01$) but not in DLB, and a trend to increased levels was noted in cortex in both disease groups ($P = 0.05$ for a 76% increase in PD vs. Controls; $P = 0.17$ for a 60% increase in DLB vs. Controls) (Fig. 2). As LCMT-1 and PME-1 signals were normalized against the number of residual melanized neurons, and PME-1 expression is increased in the SN, these findings indicate that the observed decrease in LCMT-1 expression in SN (Fig. 1) is not explained by the loss of dopaminergic neurons. Additionally, the marked decrease in LCMT-1 in the SN of both PD and DLB, despite only mild to moderate depigmentation in the latter, also suggests that neuronal loss cannot adequately account for the decrease in LCMT-1 expression in these conditions.

In agreement with the robust decrease in LCMT-1 expression in the SN of both PD and DLB brains, and increased PME-1 expression in PD, methylated PP2A expression was markedly down-regulated in SN in both conditions compared to Controls (Fig. 3). Methyl-PP2A was reduced by 70% in PD ($P < 0.001$) and by 73% in DLB ($P < 0.0001$) (Fig. 3A–D), while no significant differences were detected in the levels of demethylated PP2A (Fig. 3E–H). Total PP2A expression (Fig. 3J–M), and total B55α subunit levels were no different among the three groups (Fig. 3N–Q). The finding that the proportion of methylated PP2A out of total PP2A is decreased by 60% in PD ($P < 0.01$) and by 65% in DLB SN ($P < 0.001$) compared to Controls (Fig. 3D) indicates that the catalytically active form of PP2A is diminished due to a decrease in its methylating enzyme LCMT-1 (Fig. 1), with contribution from increased expression of

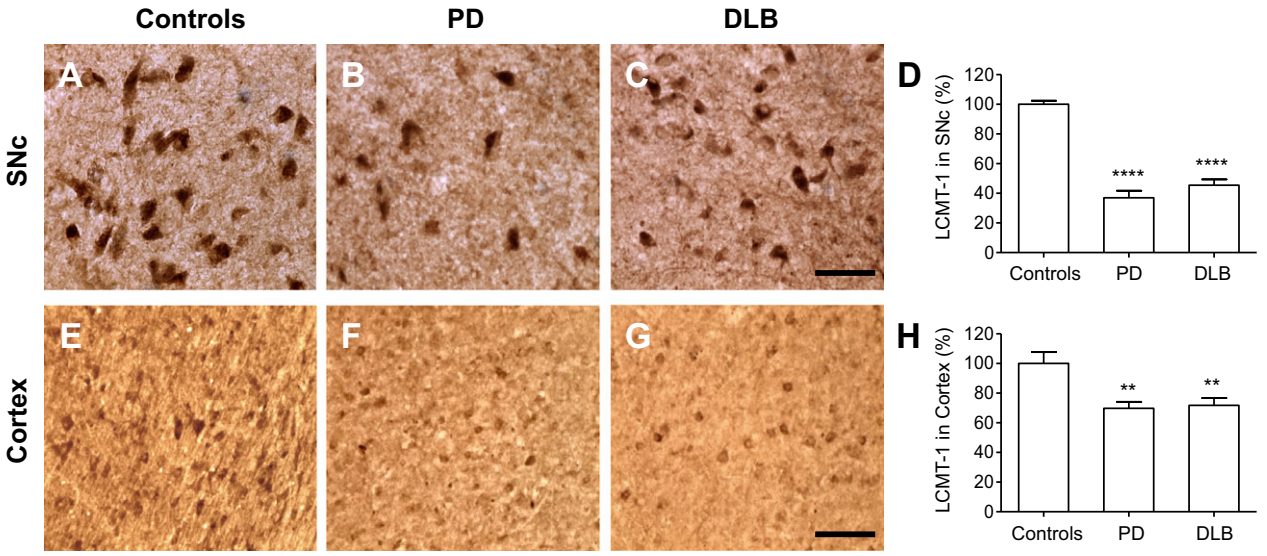

Figure 1. Comparison of LCMT-1 expression in Parkinson disease (PD), dementia with Lewy bodies (DLB), and Control brains. Tissue sections from the substantia nigra pars compacta (A–D) and middle frontal gyrus of the cerebral cortex (E–H) were immunohistochemically stained for LCMT-1. (A–C) Representative images of LCMT-1 immunoreactivity in the substantia nigra. (D) Optical density values for LCMT-1 staining intensity in the substantia nigra normalized against the number of neuromelanin-containing neurons for a sample size of eight in each group. (E–G) Representative images of LCMT-1 immunoreactivity in the cortex. (H) Optical density values for LCMT-1 staining intensity in the cortex (n = 8 in each group.) ** ANOVA $P < 0.01$; **** $P < 0.0001$ compared to Controls. Scale bar = 100 μm.

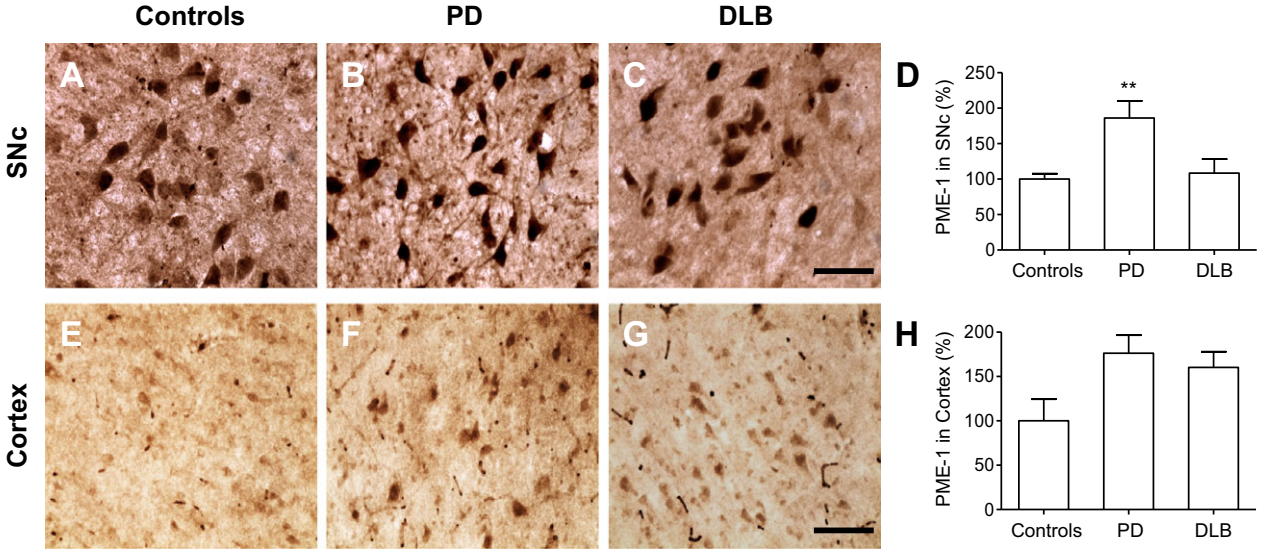

Figure 2. Comparison of PME-1 expression in Parkinson disease (PD), dementia with Lewy bodies (DLB), and Control brains. Tissue sections from the substantia nigra (A–D) and middle frontal gyrus of the cerebral cortex (E–H) were immunohistochemically stained for PME-1. (A–C) Representative images of PME-1 immunoreactivity in the substantia nigra. (D) Optical density values for PME-1 staining intensity in the nigra, normalized against the number of neuromelanin-containing neurons, in Controls (n = 8), PD (n = 7), and DLB (n = 8). ** ANOVA $P < 0.01$ compared to Controls. (E–G) Representative images of PME-1 immunoreactivity in the cortex. (H) Optical density values for PME-1 staining intensity in the cortex (n = 8 in each group.) None of the differences were significant. Scale bar = 100 μm.

the methylesterase PME-1 (Fig. 2). As all these analyses are normalized against the number of neuromelanin-containing neurons, the decrease in LCMT-1 expression represents a pathologic marker and not a reflection of dopaminergic neuronal loss in these conditions. Furthermore, the pathologic imbalance of the methylation state of PP2A is evident from the marked decrease in the ratio between methylated PP2A and demethylated PP2A in the

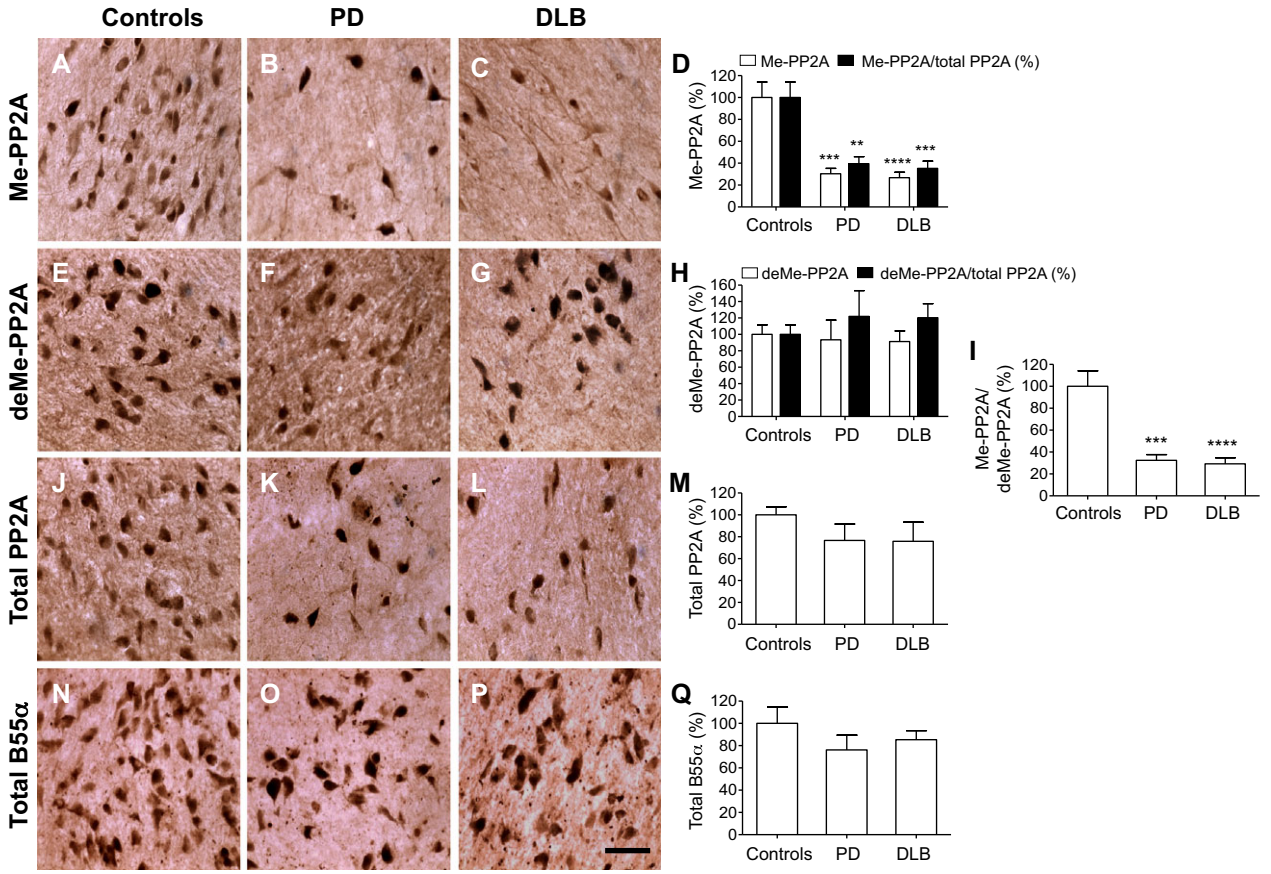

Figure 3. Immunoreactivity patterns of methylated-PP2A, demethylated-PP2A, total PP2A, and total B55α subunit in the substantia nigra of Parkinson disease (PD), dementia with Lewy bodies (DLB), and Control brains. Tissue sections from the substantia nigra were immunohistochemically stained using antibodies against methylated-PP2A (6A3), demethylated-PP2A (1D6), total PP2A (06-222), and total B55α subunit (2G9). (A–C) Representative images of methylated-PP2A immunoreactivity. (D) Optical density values for methylated-PP2A staining intensity (white bars) and its ratio to total PP2A (black bars) in Controls (n = 7), PD (n = 6), and DLB (n = 8) brains. (E–G) Representative images of demethylated-PP2A immunoreactivity. (H) Optical density values for demethylated-PP2A staining intensity (white bars) and its ratio to total PP2A (black bars) for a sample size of 8 in each group. (I) Ratio between methylated-PP2A and demethylated-PP2A for Controls (n = 7), PD (n = 6), and DLB (n = 8) brains. (J–L) Representative images of total-PP2A immunoreactivity. (M) Optical density values for total PP2A staining intensity for Controls (n = 7), PD (n = 6), and DLB (n = 7). (N–P) Representative images of total B55α immunoreactivity. Q, Optical density values for total Bα staining intensity for Controls (n = 8), PD (n = 7), and DLB (n = 8) brains. All optical density values in the SN were normalized against the number of neuromelanin-containing neurons, **ANOVA $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ compared to Controls. Scale bar = 100 μm.

SN in both PD and DLB brains (Fig. 3I). Interestingly, a case with PD and homozygous N370S GBA mutation had the lowest level of methylated PP2A. However, the small numbers of cases analyzed in this study preclude making conclusions about a possible link between genetic background and PP2A dysregulation.

In frontal cortical tissue, the decline in methyl-PP2A expression was significant only in DLB brains but not in PD brains (Fig. 4A–D). The immunoreactive signal of methyl-PP2A was decreased by 31% in DLB ($P < 0.05$), but not significantly in PD ($P = 0.18$) compared to Controls. Demethylated PP2A, on the other hand, was increased in PD cortex by 147% ($P < 0.01$) and not in DLB ($P = 0.34$) (Fig. 4E–H). Similar trends were found with the ratios of methyl-PP2A and demethyl-PP2A to total PP2A (Fig. 4D, H). As a result, the ratio of methyl-PP2A over demethyl-PP2A was significantly decreased in both PD (67%, $P < 0.0001$) and DLB cortices (57%, $P < 0.0001$) (Fig. 4I). No changes were noted in total PP2A or total B55α in either disease condition compared to Controls. These findings suggest that there is proportionately less enzymatically active methylated PP2A relative to the inactive demethylated form in cortical tissue of both advanced PD and DLB.

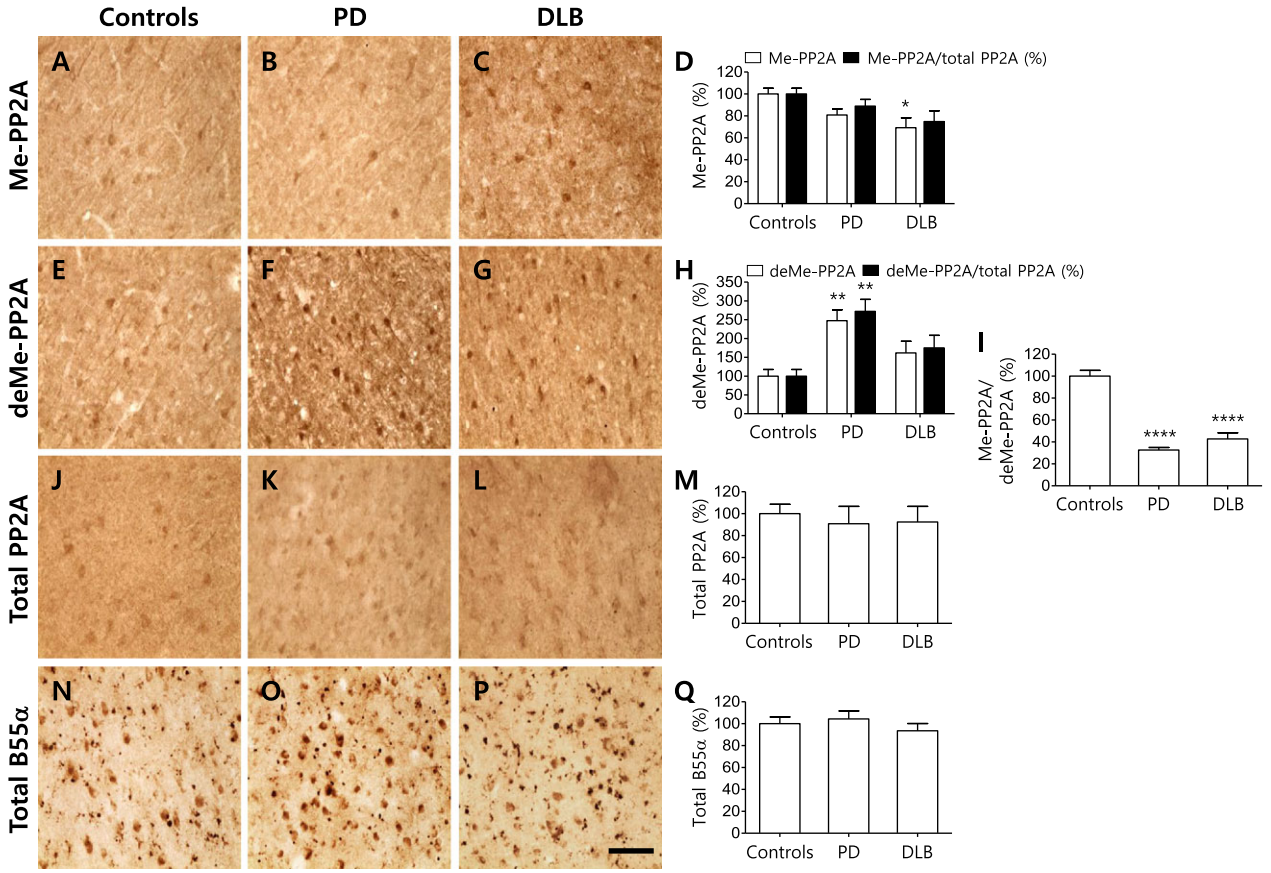

Figure 4. Immunoreactivity patterns of methylated-PP2A, demethylated-PP2A, total PP2A, and total B55α subunit in the cortex of Parkinson disease (PD), dementia with Lewy bodies (DLB), and Control brains. Tissue sections from the middle frontal gyrus of the cerebral cortex were immunohistochemically stained using antibodies against methylated-PP2A (6A3), demethylated-PP2A (1D6), total PP2A (06-222), and total B55α (2G9). (A–C) Representative images of methylated-PP2A immunoreactivity. (D) Optical density values for methylated-PP2A staining intensity (white bars) and its ratio to total PP2A (black bars) (n = 8 in each group.) (E–G) Representative images of demethylated-PP2A immunoreactivity. (H) Optical density values for demethylated-PP2A staining intensity (white bars) and its ratio to total PP2A (black bars). (I) Ratio between methylated-PP2A and demethylated-PP2A. (J–L) Representative images of total-PP2A immunoreactivity. (M) Optical density values for total PP2A staining intensity. (N–P) Representative images of total Bα immunoreactivity. (Q) Optical density values for total B55α staining intensity. For all panels, n = 8 in each group. * ANOVA $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ different from Controls. Scale bar = 100 μm.

Discussion

This study demonstrates that in the brains of individuals with the α-synucleinopathies PD and DLB, PP2A is in a state that does not favor its assembly into an enzymatically active AB55αC heterotrimeric form. Since the main dephosphorylating enzyme of α-synuclein is the B55α containing heterotrimeric isoform of PP2A,[13] and methylation of the C subunit of PP2A enhances the incorporation of the regulatory B55α subunit into the catalytically active holoenzyme,[3–5] the markedly decreased expression of the methylating enzyme LCMT-1 in these conditions, and increased expression of the demethylating enzyme PME-1, lead to a striking decrease in the methylated form of PP2A. These dysregulatory changes are not only particularly prominent in the substantia nigra pars compacta but also present in the cortex.

The impact of these maladaptive changes in the brain is profound considering that PP2A is the master regulator of the cellular phospho-regulatory network and plays key roles in regulating cytoskeletal integrity and signal transduction.[26–28] Considering the deleterious consequences of a dysfunctional PP2A, it is unlikely that the changes observed in these postmortem brain analyses represent a protective effect in surviving neurons. In relation to α-synucleinopathies in particular, PP2A catalyzes the dephosphorylation of phospho-Ser_{129} α-synuclein, and the methylation state of the PP2A C subunit regulates this activity.[13] Accordingly, the finding of decreased PP2A methylation in this study provides a molecular

mechanism for the reduced PP2A activity reported in α-synucleinopathies [16] and is likely a significant contributor to the hyperphosphorylation of aggregated α-synuclein in these disorders.[10,29]

The striking association between decreased levels of PP2A methylation and decreased levels of LCMT-1 suggests that a primary regulatory locus for PP2A methylation is LCMT-1. In assessing the regulatory parameters that might control PP2A carboxyl methylation in the CNS, there has been a focus on factors that underlie methylation metabolism such as levels of the methyl donor, S-adenosylmethionine (SAM), the competitive inhibitor, S-adenosyl homocysteine (SAH), or the methylation cycle intermediate homocysteine. Accordingly, there is considerable evidence that SAM and SAH levels play a substantial role in regulating PP2A.[30–34] Conditions of methylation and one carbon stress have been shown to cause DNA hypomethylation at loci that appear to contribute to the pathogenesis of PD.[35–37] Similar epigenetic mechanisms may function directly or indirectly to reduce the expression of LCMT-1 and increase the expression of PME-1. This might provide a transcriptional mechanism to enforce the regulatory consequences of one-carbon/methylation stress on PP2A activity, and at the same time, conserve methyl groups under conditions of methyl deficiency. In this regard, it is worth noting that L-dopa therapy appears to exacerbate methylation deficiencies and results in hyperhomocysteinemia due to the action of catechol-O-methyl transferase (COMT).[38,39]

Considerable evidence suggests that oxidative stress plays a critical role in the pathogenesis of PD [40] and DLB.[41] Evidence has also been reported that PP2A phosphatase activity is sensitive to oxidative stress,[42] and this sensitivity is proposed to be mediated by oxidation of a pair of vicinal cysteines located near the phosphatase active site of the C subunit.[43] Additionally, PP2A oxidation by exposure to micromolar levels of hydrogen peroxide has been shown to completely block its methylation by LCMT-1.[44] Furthermore, the X-ray crystal structure of a complex between LCMT-1 and PP2A supports the notion that the formation of a disulfide crosslink between these cysteine residues might preclude formation of a productive LCMT-1-PP2A complex and, thereby, block the methyltransferase activity of LCMT-1.[44] Thus, the oxidative stress environment in α-synucleinopathies may contribute to the decreased methylation of PP2A and its activity.

Besides the mechanisms that can reduce PP2A methylation, several additional factors would be expected to contribute to the state of PP2A dysregulation in α-synucleinopathies. Pathologic α-synuclein accumulation itself may be an inhibitor of PP2A activity resulting in a vicious cycle exacerbating the hyperphosphorylation and aggregation of pathogenic proteins including α-synuclein and tau in Lewy body disease.[10,45] Overexpression of α-synuclein, particularly its A53T mutant which causes dominantly inherited PD and dementia, increases intracellular levels of reactive oxygen species in SH-SY5Y neuroblastoma cells ,[46] contributing to oxidative stress-mediated dysregulation of PP2A.[42,44] Accordingly, A53T-mutant α-synuclein reportedly reduces PP2A activity more than the wild-type isoform does.[47] And in the rat striatum, α-synuclein overexpression using viral vector-mediated gene transfer results in decreased expression of PP2A B and C subunits as well as decreased PP2A activity. This effect is associated with increased α-synuclein phosphorylation and aggregation as well as neuronal cell death and inflammation.[48] Interestingly, parkin, which is linked to recessively inherited PD due to loss of function mutations, reportedly prevents α-synuclein-mediated reduction in PP2A expression and activity and mitigates the pathology induced by α-synculein.[48] The negative impact of impaired PP2A activity is also exerted on parkin functioning as well contributing to neuronal dyshomeostasis. Inhibition of PP2A with okadaic acid in cultured primary neurons reduces parkin expression level and K48-linked polyubiquitination, which is implicated in parkin-mediated mitophagy, suggesting that reduced PP2A activity impairs the machinery necessary for mitophagy leading to the accumulation of damaged mitochondria.[49]

Another mechanism through which α-synuclein accumulation leads to impaired PP2A activity is through the lysosomal enzyme glucocerebrosidase (GCase), which has strong genetic link to PD and is decreased in sporadic PD brains.[50,51] In this regard, accumulation of oligomeric and phosphorylated α-synuclein with age in the brains of cynomolgus monkeys is associated with decreased expression and activity of GCase, as well as reduced activity of PP2A particularly in brain regions that are susceptible to α-synucleinopathy-related neurodegeneration. This inverse association between GCase activity, α-synuclein phosphorylation, and PP2A activity is also demonstrated in cultured neuronal cells, whereby inhibition of GCase activity leads to increased α-synuclein phosphorylation and reduced methylated PP2A levels and activity.[52,53] Inhibition of autophagy due to loss of GCase function is also associated with inactivation of PP2A,[53] while activation of autophagy with the mTOR inhibitor rapamycin or metformin stimulates PP2A and reduces α-synuclein phosphorylation in neuronal cells.[54–56] GCase dysfunction may reduce PP2A activity through impaired lysosomal function and consequent α-synuclein accumulation. In addition, GCase can also modulate PP2A directly through the product of its enzymatic activity, ceramide, which is an activator of PP2A [57,58] and of autophagy.[53] Thus, a reduction in GCase activity decreases the production of

ceramide leading to reduced PP2A activity. This may explain why accumulation of α-synuclein in GCase mutants can be reversed by ceramide.[53]

The present findings point to parallels in the dysregulation of PP2A, and in particular its methylation state, between α-synucleinopathies and Alzheimer's disease.[34] Decreased phosphatase activity toward abnormally phosphorylated tau had been recognized in AD brains.[59] Evidence for the mechanism of this decrease in phosphatase activity comes from the finding that LCMT-1 is decreased in AD brains, associated with decreased levels of methylated PP2A, and closely matching tau pathology.[17] In addition, levels of Bα containing PP2A critical for regulating tau [60] are decreased in tangle-bearing neurons correlating with increased tau pathology.[18] And the sensitivity to exogenously administered Aβ oligomers is reduced in the brains of transgenic animals overexpressing LCMT-1 and exacerbated in mice overexpressing PME-1.[61] In this study, although DLB cases have more advanced concomitant AD-like pathology than PD cases (Table 1), the observation that indices of PP2A dysregulation in the cortex are not worse in DLB compared to PD suggests that α-synucleinopathy is the primary driver of PP2A dysregulation in these two conditions. The finding that downregulation of LCMT-1 and changes in PP2A methylation are more prominent in the SN compared to the cortex in PD and DLB supports this notion and may be due to greater oxidative stress environment in the SN. Nevertheless, the contribution of concomitant AD pathology to these changes cannot be entirely excluded. Among the neurobiological consequences of altered LCMT-1 expression and PP2A methylation that relate to neurodegenerative diseases is neuritogenesis. Experimentally, in cultured neuroblastoma cells, enhanced expression of LCMT-1 increases methylated C and Bα levels and promotes neuritogenesis.[62] These findings collectively suggest a global role of methylated PP2A in regulating brain protein homeostasis and neuronal survival in neurodegenerative diseases.

The above findings from postmortem brain analysis of PD and DLB patients suggest that efforts to enhance PP2A activity are a plausible therapeutic strategy in order to mitigate the neuropathology of α-synucleinopathies. The ability of EHT, which prevents the demethylation of PP2A, in reducing α-synuclein phosphorylation and aggregation associated with improved neuropathological abnormalities and behavioral deficits in α-synuclein transgenic mice [13] supports this conclusion. Enhancing PP2A activity has also been shown to mitigate the pathology in an AD model as well: EHT treatment resulted in substantial amelioration of AD-like pathologies such as tau hyperphosphorylation, elevated amyloid-β levels, and cognitive impairment in a rat model of AD generated by viral vector-mediated expression of the PP2A endogenous inhibitor $I2^{PP2A}$, or SET protein, in the brain.[19] And in aged mice and in transgenic tauopathy models of AD, the use of another enhancer of PP2A activity, sodium selenite, has also been demonstrated to reduce tau hyperphosphorylation.[63,64] Thus, PP2A modulation may provide a common approach for treating neurodegenerative proteinopathies associated with hyperphosphorylated pathogenic proteins.

Acknowledgment

We are grateful to the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona, and the Arizona Parkinson Disease consortium for the clinical data and provision of human brain tissue samples.

This study was supported by a grant from the Michael J. Fox Foundation for Parkinson's Research to S.P.B. and M.M.M. T.G.B. is supported by grants from the NIA, NINDS, Michael J. Fox Foundation and the State of Arizona. J.B.S. is supported by NIH grant AT006868. M.M.M. is the William Dow Lovett Professor of Neurology and is supported by the American Parkinson Disease Association and by NIH grants AT006868 and NS073994. The Banner Sun Health Research Institute Brain and Body Donation Program is supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson's Disease and Related Disorders), the National Institute on Aging (P30 AG19610 Arizona Alzheimer's Disease Core Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05-901 and 1001 to the Arizona Parkinson's Disease Consortium), and the Michael J. Fox Foundation for Parkinson's Research.

Author contribution

Experiments were conceived by M.V., S.P.B., J.B.S., and M.M.M. Experiments were designed by H.-J., P., K.W.L. and M.M.M. Brain sections, clinical and neuropathological information were contributed by T.G.B. and C.H.A. Experiments were performed by H.-J. P., K.W.L., and E.S.P. Data were analyzed by H.-J. P., S.O., R.Y., J.Z., and M.M.M. Results were interpreted by M.V., S.P.B., J.B.S., and M.M.M. The manuscript was drafted by H.-J.P. T.G.B., C.H.A., M.V., S.P.B., J.B.S. and M.M.M. All authors critically reviewed the manuscript and approved the final version.

Conflict of Interest

J.B.S has a significant financial interest in Signum Biosciences, which is developing PP2A phosphatase-enhancing agents. M.V. is employed by Signum Biosciences. S.P.B. was employed by Signum Biosciences. T.G.B. has received personal fees from GE Healthcare and Avid Radiopharmaceuticals for work unrelated to this study. Other co-authors have no relevant potential conflict of interest to report.

References

1. Strack S, Westphal RS, Colbran RJ, et al. Protein serine/threonine phosphatase 1 and 2A associate with and dephosphorylate neurofilaments. Brain Res Mol Brain Res 1997;49:15–28.
2. Strack S, Zaucha JA, Ebner FF, et al. Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. J Comp Neurol 1998;392:515–527.
3. Bryant JC, Westphal RS, Wadzinski BE. Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. Biochem J 1999;339(Pt 2):241–246.
4. Tolstykh T, Lee J, Vafai S, Stock JB. Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. EMBO J 2000;19:5682–5691.
5. Wu J, Tolstykh T, Lee J, et al. Carboxyl methylation of the phosphoprotein phosphatase 2A catalytic subunit promotes its functional association with regulatory subunits in vivo. EMBO J 2000;19:5672–5681.
6. Lee J, Chen Y, Tolstykh T, Stock J. A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain. Proc Natl Acad Sci U S A 1996;93:6043–6047.
7. Lee J, Stock J. Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase. J Biol Chem 1993;268:19192–19195.
8. Ogris E, Du X, Nelson KC, et al. A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. J Biol Chem 1999;274:14382–14391.
9. Spillantini MG, Schmidt ML, Lee VM, et al. Alpha-synuclein in Lewy bodies. Nature 1997;388:839–840.
10. Fujiwara H, Hasegawa M, Dohmae N, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002;4:160–164.
11. Neumann M, Kahle PJ, Giasson BI, et al. Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. J Clin Invest 2002;110:1429–1439.
12. Anderson JP, Walker DE, Goldstein JM, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. J Biol Chem 2006;281:29739–29752.
13. Lee KW, Chen W, Junn E, et al. Enhanced phosphatase activity attenuates alpha-synucleinopathy in a mouse model. J Neurosci 2011;31:6963–6971.
14. Chen L, Feany MB. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. Nat Neurosci 2005;8:657–663.
15. Gong CX, Lidsky T, Wegiel J, et al. Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. J Biol Chem 2000;275:5535–5544.
16. Wu J, Lou H, Alerte TN, et al. Lewy-like aggregation of alpha-synuclein reduces protein phosphatase 2A activity in vitro and in vivo. Neuroscience 2012;207:288–297.
17. Sontag E, Hladik C, Montgomery L, et al. Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. J Neuropathol Exp Neurol 2004;63:1080–1091.
18. Sontag E, Luangpirom A, Hladik C, et al. Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology. J Neuropathol Exp Neurol 2004;63:287–301.
19. Basurto-Islas G, Blanchard J, Tung YC, et al. Therapeutic benefits of a component of coffee in a rat model of Alzheimer's disease. Neurobiol Aging 2014;35:2701–2712.
20. Beach TG, Adler CH, Sue LI, et al. Arizona Study of Aging and Neurodegenerative Disorders and Brain and Body Donation Program. Neuropathology 2015;35:354–389.
21. Beach TG, Adler CH, Lue L, et al. Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. Acta Neuropathol 2009;117:613–634.
22. Iacono D, Geraci-Erck M, Rabin ML, et al. Parkinson disease and incidental Lewy body disease: Just a question of time? Neurology 2015;85:1670–1679.
23. Mirra SS, Heyman A, McKeel D, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991;41:479–486.
24. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 1991;82:239–259.
25. Wei H, Ashby DG, Moreno CS, et al. Carboxymethylation of the PP2A catalytic subunit in Saccharomyces cerevisiae is required for efficient interaction with the B-type

subunits Cdc55p and Rts1p. J Biol Chem 2001;276:1570–1577.
26. Sontag E. Protein phosphatase 2A: the Trojan Horse of cellular signaling. Cell Signal 2001;13:7–16.
27. Shi Y. Serine/threonine phosphatases: mechanism through structure. Cell 2009;139:468–484.
28. Sangodkar J, Farrington C, McClinch K, et al. All roads lead to PP2A: Exploiting the therapeutic potential of this phosphatase. FEBS J 2016; 283:1004–1024.
29. Ikeuchi T, Kakita A, Shiga A, et al. Patients homozygous and heterozygous for SNCA duplication in a family with parkinsonism and dementia. Arch Neurol 2008;65:514–519.
30. Vafai SB, Stock JB. Protein phosphatase 2A methylation: a link between elevated plasma homocysteine and Alzheimer's Disease. FEBS Lett 2002;518:1–4.
31. Sontag E, Nunbhakdi-Craig V, Sontag JM, et al. Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation. J Neurosci 2007;27:2751–2759.
32. Obeid R, Schlundt J, Umanskaya N, et al. Folate is related to phosphorylated neurofilament-H and P-tau (Ser396) in rat brain. J Neurochem 2011;117:1047–1054.
33. Gueant JL, Caillerez-Fofou M, Battaglia-Hsu S, et al. Molecular and cellular effects of vitamin B12 in brain, myocardium and liver through its role as co-factor of methionine synthase. Biochimie 2013;95:1033–1040.
34. Sontag JM, Sontag E. Protein phosphatase 2A dysfunction in Alzheimer's disease. Front Mol Neurosci 2014;7:16.
35. Kaut O, Schmitt I, Wullner U. Genome-scale methylation analysis of Parkinson's disease patients' brains reveals DNA hypomethylation and increased mRNA expression of cytochrome P450 2E1. Neurogenetics 2012;13:87–91.
36. Feng Y, Jankovic J, Wu YC. Epigenetic mechanisms in Parkinson's disease. J Neurol Sci 2015;349:3–9.
37. Pihlstrom L, Berge V, Rengmark A, Toft M. Parkinson's disease correlates with promoter methylation in the alpha-synuclein gene. Mov Disord 2015;30:577–580.
38. Postuma RB, Lang AE. Homocysteine and levodopa: should Parkinson disease patients receive preventative therapy? Neurology 2004;63:886–891.
39. Bottiglieri T, Arning E, Wasek B, et al. Acute administration of L-DOPA induces changes in methylation metabolites, reduced protein phosphatase 2A methylation, and hyperphosphorylation of Tau protein in mouse brain. J Neurosci 2012;32:9173–9181.
40. Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. J Parkinsons Dis 2013;3:461–491.
41. Mao P. Oxidative Stress and Its Clinical Applications in Dementia. J Neurodegener Dis. 2013;2013:319898.
42. Rao RK, Clayton LW. Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation. Biochem Biophys Res Commun 2002;293:610–616.
43. Foley TD, Melideo SL, Healey AE, et al. Phenylarsine oxide binding reveals redox-active and potential regulatory vicinal thiols on the catalytic subunit of protein phosphatase 2A. Neurochem Res 2011;36:232–240.
44. Stanevich V, Jiang L, Satyshur KA, et al. The structural basis for tight control of PP2A methylation and function by LCMT-1. Mol Cell 2011;41:331–342.
45. Ishizawa T, Mattila P, Davies P, et al. Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. J Neuropathol Exp Neurol 2003;62:389–397.
46. Junn E, Mouradian MM. Human alpha-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. Neurosci Lett 2002;320:146–150.
47. Perfeito R, Lazaro DF, Outeiro TF, Rego AC. Linking alpha-synuclein phosphorylation to reactive oxygen species formation and mitochondrial dysfunction in SH-SY5Y cells. Mol Cell Neurosci 2014;62:51–59.
48. Khandelwal PJ, Dumanis SB, Feng LR, et al. Parkinson-related parkin reduces alpha-Synuclein phosphorylation in a gene transfer model. Mol Neurodegener 2010;5:47.
49. Cho MH, Kim DH, Choi JE, et al. Increased phosphorylation of dynamin-related protein 1 and mitochondrial fission in okadaic acid-treated neurons. Brain Res 2012;1454:100–110.
50. Sidransky E, Nalls MA, Aasly JO, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N Engl J Med 2009;361:1651–1661.
51. Gegg ME, Burke D, Heales SJ, et al. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Ann Neurol 2012;72:455–463.
52. Liu G, Chen M, Mi N, et al. Increased oligomerization and phosphorylation of alpha-synuclein are associated with decreased activity of glucocerebrosidase and protein phosphatase 2A in aging monkey brains. Neurobiol Aging 2015;36:2649–2659.
53. Du TT, Wang L, Duan CL, et al. GBA deficiency promotes SNCA/alpha-synuclein accumulation through autophagic inhibition by inactivated PPP2A. Autophagy 2015;11:1803–1820.
54. Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc Natl Acad Sci U S A 1999;96:4438–4442.
55. Hartley D, Cooper GM. Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. J Cell Biochem 2002;85:304–314.
56. Perez-Revuelta BI, Hettich MM, Ciociaro A, et al. Metformin lowers Ser-129 phosphorylated alpha-synuclein levels via mTOR-dependent protein phosphatase 2A activation. Cell Death Dis 2014;5:e1209.

57. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. J Biol Chem 1993;268:15523–15530.
58. Chalfant CE, Kishikawa K, Mumby MC, et al. Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. J Biol Chem 1999;274:20313–20317.
59. Gong CX, Shaikh S, Wang JZ, et al. Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. J Neurochem 1995;65: 732–738.
60. Sontag E, Nunbhakdi-Craig V, Lee G, et al. Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J Biol Chem 1999;274:25490–25498.
61. Nicholls RE, Sontag JM, Zhang H, et al. PP2A methylation controls sensitivity and resistance to beta-amyloid-induced cognitive and electrophysiological impairments. Proc Natl Acad Sci U S A 2016; 113:3347–3352.
62. Sontag JM, Nunbhakdi-Craig V, Mitterhuber M, et al. Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells. J Neurochem 2010;115:1455–1465.
63. Corcoran NM, Martin D, Hutter-Paier B, et al. Sodium selenate specifically activates PP2A phosphatase, dephosphorylates tau and reverses memory deficits in an Alzheimer's disease model. J Clin Neurosci 2010;17:1025–1033.
64. van Eersel J, Ke YD, Liu X, et al. Sodium selenate mitigates tau pathology, neurodegeneration, and functional deficits in Alzheimer's disease models. Proc Natl Acad Sci U S A. 2010;107:13888–13893.

