α-Synuclein Sequesters Dnmt1 from the Nucleus

A NOVEL MECHANISM FOR EPIGENETIC ALTERATIONS IN LEWY BODY DISEASES

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DNA methylation is a major epigenetic modification that regulates gene expression. Dnmt1, the maintenance DNA methylation enzyme, is abundantly expressed in the adult brain and is mainly located in the nuclear compartment, where it has access to chromatin. Hypomethylation of CpG islands at intron 1 of the SNCA gene has recently been reported to result in overexpression of α-synuclein in Parkinson disease (PD) and related disorders. We therefore investigated the mechanisms underlying altered DNA methylation in PD and dementia with Lewy bodies (DLB). We present evidence of reduction of nuclear Dnmt1 levels in human postmortem brain samples from PD and DLB patients as well as in the brains of α-synuclein transgenic mice models. Furthermore, sequestration of Dnmt1 in the cytoplasm results in global DNA hypomethylation in human and mouse brains, involving CpG islands upstream of SNCA, SEPW1, and PRKAR2A genes. We report that association of Dnmt1 and α-synuclein might mediate aberrant subcellular localization of Dnmt1. Nuclear Dnmt1 levels were partially rescued by overexpression of Dnmt1 in neuronal cell cultures and in α-synuclein transgenic mice brains. Our results underscore a novel mechanism for epigenetic dysregulation in Lewy body diseases, which might underlie the decrease in DNA methylation reported for PD and DLB.

Parkinson disease (PD) and dementia with Lewy bodies (DLB) belong to a heterogeneous group of neurodegenerative disorders known as Lewy body diseases, which are characterized by α-synuclein (α-syn) accumulation in Lewy body structures (1, 2). The most striking pathological feature of PD is the loss of dopaminergic neurons in the substantia nigra, although neurodegeneration has been also reported to occur in the cortex. In DLB, non-motor cognitive alterations appear before parkinsonism and α-syn accumulates mainly in the limbic system and neocortical areas, suggesting that these structures have an important role in disease (3). At the molecular level, accumulation of misfolded α-syn affects mitochondrial function, autophagy (4), and the expression of several functional groups of genes (5, 6), although the molecular basis of transcriptional dysregulation remain elusive.

Proper gene expression is crucial for the cell, and therefore, it is tightly regulated by the binding of regulatory proteins to promoter regions and by the epigenetic remodeling of the chromatin structure by DNA methylation and histone modifications. Although the traditional concept of epigenetics refers to stable and heritable changes associated with cell division and development, there is mounting evidence supporting that DNA methylation is dynamically regulated in postmitotic neurons, having crucial roles in memory formation and synaptic plasticity (7). Altered epigenetic mechanisms have also been associated with neurological disorders, including Rett syndrome, autism, schizophrenia, and Alzheimer, Huntington, and Parkinson diseases (8). The relevance of DNA methylation in PD pathology was highlighted recently by two studies showing that decreased DNA methylation at intron 1 of the human SNCA gene might directly contribute to dysregulation of α-syn expression in sporadic PD cases (9, 10).

In the present study, we investigated whether expression of DNA methyltransferase 1 (Dnmt1) might be altered during PD and DLB pathology, thus resulting in hypomethylation of DNA. We present evidence of mislocalization of Dnmt1 in the cytoplasm of neuronal cells overexpressing α-syn in postmortem human brain samples from PD and DLB patients as well as in a mouse model of PD/DLB. Moreover, we show that association of Dnmt1 with α-syn might mediate the retention of Dnmt1 in the cytoplasm, an effect that could be at least partially reversed in vivo by lentivirus-mediated overexpression of Dnmt1.

EXPERIMENTAL PROCEDURES

Human Brain Samples—Postmortem human brain samples (frontal cortex) were provided by the Alzheimer’s Disease Research Center at University of California San Diego (UCSD) from patients diagnosed with PD (n = 4) and DLB (n = 4) and from non-demented age-matched control subjects (n = 4), postmortem interval ≤ 8 h. All cases were clinically characterized during life and histopathologically characterized postmortem.

Animal Models—Experiments were conducted on 8-month-old transgenic mice expressing wild type human α-syn (α-syn tg) under the Thy-1 promoter (11) and on age- and sex-matched control wild type animals (n = 4/group). When indicated, mice were injected with 3 μl of the lentiviral preparation

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1 and 2.
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[3] The abbreviations used are: PD, Parkinson disease; DBL, dementia with Lewy bodies; α-syn, α-synuclein; β-syn, β-synuclein; GFAP, glial fibrillary acidic protein; tg, transgenic; 5′-mC, 5-methyl-cytosine.
Dnmt1 Exclusion from the Nucleus in PD/DLB

(2.5 × 10^7 transducing units) into the hippocampus as described previously (12). Brains were extracted after cervical dislocation and immediately frozen or fixed until processing. All animals were handled in strict accordance with good animal practice, under the specifications set forth by the UCSD Institutional Animal Care and Use Committee.

Lentivirus Production—Lentivirus vectors expressing human α-syn (LV-α-syn), human β-synuclein (β-syn) (LV-β-syn), empty cassette (LV-control), and human Dnmt1 (LV-Dnmt1) and Myc epitope-tagged Dnmt1 (LV-Dnmt1 Myc) (both from GeneCopoeia) were prepared by transient transfection in 293T cells as described elsewhere (13).

Cell Culture—Rat B103 neuroblastoma cells and 293T human hepatocarcinoma cells were grown as described previously (12). Cells were infected with lentivirus at a multiplicity of infection of 15 for 48 h and fixed with paraformaldehyde.

Nuclear and Cytoplasmic Protein Extraction—Nuclear and cytoplasmic fractions were obtained from 1 × 10^6 cells or 100 mg of human or mouse brain tissue with the EpiQuik nuclear extraction kit (Epigentek). Protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific).

DNA Extraction and Global DNA Methylation Assays—Genomic DNA was extracted from 1 × 10^6 cells or 25 mg of human or mouse brain tissue with the DNeasy blood and tissue kit (Qiagen). DNA methylation was measured in 200 ng of genomic DNA with the Methylamp global DNA methylation quantification ultra kit (Epigentek). Each sample was run in triplicate.

Methylation-specific PCR—Human genomic DNA (1 μg) was modified with sodium bisulfite (EpiTect kit, Qiagen). 100 ng of converted DNA was amplified with the EpiTect MSP kit (Qiagen) using specific methylated or unmethylated primers (supplemental Table 1) designed with Methyl Primer Express version 1.0 (Applied Biosystems) and following the cycling conditions indicated by the vendor.

Detection and Quantification of Dnmt1—Electrophoretic analysis and Western blotting detection of target proteins were performed as described previously (14), using the following antibodies: Dnmt1 (1:500, Abcam), α-syn (1:1,000, Millipore Bioscience Research Reagents), β-syn (1:1,000, BD Transduction Laboratories), or β-actin (1:1,000, Millipore). The specificity of the ~183-KDa band detected by anti-Dnmt1 antibody was confirmed by using the corresponding blocking peptide from Abcam (1:1.5) to preadsorb the antibody. ELISA quantification of Dnmt1 was performed in 20 μg of protein homogenate with the EpiQuik Dnmt1 assay kit (Epigentek).

Immunohistochemical Detection of Dnmt1 and DNA Methylation—Paraffin-embedded postmortem human cortical sections, free-floating sections from mouse brains, or cells grown on coverslips were processed as described previously (14) with the following antibodies: Dnmt1 (1:250, Abcam), α-syn (1:350, Millipore or BD Biosciences), anti-Myc (1:250, Santa Cruz Biotechnology), β-syn (1:350, BD Transduction Laboratories), and GFAP astrocytic marker or NeuN neuronal marker (both 1:5,000, Millipore). DNA methylation was quantified in brain sections with anti-5-methyl-C (1:500, Calbiochem).

Co-immunoprecipitation of Target Proteins—Total protein homogenates (600 μg) were immunoprecipitated with 2 μg of the indicated antibodies or rabbit or mouse IgGs (both from Santa Cruz Biotechnology) bound to Dynabeads protein A magnetic beads (Invitrogen) as described previously (15).

Statistical Analysis—Data represent mean values ± S.E. from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey’s multiple comparison test or Student’s t test (unpaired; two-tailed) with a significance of p < 0.05 (Prism Graph Pad Software).

RESULTS

Expression of Dnmt1 Is Decreased in the Brains of PD and DLB Patients—Dnmt1 is the maintenance methylation enzyme, which preserves the methylation patterns established early in development. Dnmt1 is abundantly expressed in the adult brain (16) and constitutes a candidate target to study the mechanisms of aberrant DNA methylation associated with aging. We investigated the expression levels of Dnmt1 in postmortem human brain samples from PD and DLB patients. ELISA assays showed a pronounced decrease in Dnmt1 protein in total homogenates from the cortex of both PD and DLB cases when compared with controls (Fig. 1A). These results were further confirmed by Western blot analysis that showed a reduction of almost 50% in nuclear Dnmt1 in PD and DLB brains (Fig. 1, B and C).

Overexpression of α-syn Results in Cytoplasmic Retention of Dnmt1—In addition to Dnmt1 levels, we quantified monomeric and oligomeric α-syn in the studied cases (Fig. 1B). As expected, increased aggregation of α-syn was detected in PD and DLB samples, evidenced by the appearance of higher molecular weight species at the expense of monomeric forms. We observed an inverse trend between oligomerization of α-syn and reduction of nuclear Dnmt1, suggesting that subcellular localization of Dnmt1 might be altered by α-syn accumulation and/or aggregation. Immunohistochemical analysis of postmortem human brain sections revealed dramatic changes in the subcellular localization of Dnmt1, which appeared mostly in the cytoplasm of PD and DLB brain cells, whereas discrete nuclear immunoreactivity was observed in the control cases (Fig. 1, D and E). Dnmt1 signal co-localized with the neuronal marker NeuN, but not with the astrocytic marker GFAP, suggesting that mislocalization of Dnmt1 is restricted to neuronal cells (Fig. 1F).

DNA Is Hypomethylated in PD and DLB Brains—The dramatic decrease in nuclear Dnmt1 is expected to impact DNA methylation. We investigated the status of global DNA methylation on human brain tissue sections with an antibody recognizing 5-methyl-cytosine (5-mC). Human cortex samples from PD and DLB patients showed at least a 2-fold reduction in 5-mC immunoreactive nuclei (Fig. 2, A and B). Moreover, the co-localization of nuclear Dnmt1 and 5-mC showed a 6-fold reduction in PD and DLB brains, indicating that mislocalization of this methylase directly alters DNA methylation (Fig. 2, C and D). In agreement with these results, a decrease of up to 30% in global DNA methylation was detected by ELISA in the diseased groups (Fig. 2E).
The methylation status of CpG islands at the 5'-regulatory regions of genes directly affects transcription. To evaluate whether the reduction in DNA methylation in PD and DLB brains might be associated with gene regulatory regions, we investigated the methylation status of CpG islands upstream of SNCA, SEPW1, and PRKAR2A genes in control, PD, and DLB samples by methylation-sensitive PCR (Fig. 2, F and G). Previous studies showed hypomethylation at intron 1 of the SNCA gene in PD brains (9, 10). In line with these reports, we found similar reductions of methylation at SNCA intron 1 in our PD samples. Moreover, we detected a comparable decay in methylation in DLB samples, suggesting that similar epigenetic dysregulation might operate in related Lewy body pathologies. Additionally, we investigated the methylation of SEPW1 and PRKAR2A regulatory regions because both genes were found to be up-regulated in PD brains in a comprehensive microarray study (17) and are expected to be hypomethylated. We found significant decreases in methylation for SEPW1 only in the DLB group, whereas PRKAR2A promoter was hypomethylated only in PD samples, indicating that specific genes might be dysregulated in each disorder. Taken together, this evidence suggests that mislocalization of Dnmt1 and reduction of DNA methylation might directly affect gene expression.

Dnmt1 Is Associated with α-syn and Is Sequestered in the Cytoplasm—To investigate possible mechanisms of cytoplasmic retention of Dnmt1, we established a cellular model in rat B103 neuronal cells by lentivirus-mediated overexpression of wild type human α-syn (Fig. 3). Although infection of the cells with control lentivirus did not affect the subcellular distribution of Dnmt1, cells infected with LV-α-syn showed a shift of Dnmt1 from the nucleus to the cytoplasmic compartment, thus reproducing our observations in human tissues (Fig. 3 A). Dnmt1 immunoreactivity was localized to the nucleus in LV-control cells, where it appeared as a punctate signal, as this protein is known to associate with replication foci in cells undergoing division. In contrast, in LV-α-syn-infected cells, Dnmt1 was sequestered in the cytoplasm (Fig. 3, C and E).

We investigated whether mislocalization of Dnmt1 was a specific event mediated by α-syn and related to its misfolding/aggregation by testing the effects of β-syn on Dnmt1 subcellular localization. α- and β-synucleins are highly homologous molecules expressed in neocortical areas and related to PD and DLB pathologies (18); however, β-syn is not found in Lewy bodies and does not aggregate to form amyloid fibrils. Lentivirus-mediated overexpression of β-syn in B103 cells did not alter the nuclear localization of Dnmt1, and similar patterns of punctuated immunoreactivity were obtained for both LV-control-infected and LV-β-syn-infected cells, suggesting that cytoplasmic sequestration of Dnmt1 is specifically linked to α-syn (Fig. 3, D and F).

The previous findings suggest that association of Dnmt1 and α-syn might alter the proper shuttling of Dnmt1 into the nucleus. To investigate this hypothesis, we performed immunoprecipitation assays in cells and human brains. Lentiviral
constructs expressing Myc-tagged human α-syn or human Dnmt1 were used to co-infect 293T cells. Protein complexes immunoprecipitated with anti-Dnmt1 antibody revealed the presence of Myc-α-syn, whereas the reverse immunoprecipitation with anti-Myc antibody showed the presence of Dnmt1 (~183 kDa) in Western blot analysis (Fig. 3G). To determine whether this interaction holds true for the endogenous proteins, we performed similar experiments on postmortem human samples. Brain homogenates from control, PD, and DLB cases were immunoprecipitated with anti-α-syn antibody showed the presence of Dnmt1 (Fig. 3H), thus supporting the in vivo association of these proteins. Concordantly with our

FIGURE 2. DNA methylation is reduced in PD and DLB brains. A, immunodetection of 5-mC in human brain sections of CT (control), PD, and DLB cases (arrows, neuronal nuclei). Bar represents 10 μm. B, image analysis showing corrected optical density per group. C, co-localization of nuclear Dnmt1 and 5-mC (arrows, neuronal nuclei; arrowheads, neuronal cells). Bar represent 20 μm. D, image analysis showing the percentage of total nuclei positive for 5-mC. E, quantification of DNA methylation in postmortem human cortex samples by ELISA. F, MSP analysis of the methylation status of CpG islands upstream of the SNCA, SEPW1, and PRKAR2A genes. Bisulfite-converted DNA from control, PD, or DLB brains was amplified using specific primers to detect methylated (M) or unmethylated CpGs (no M). G, image analysis showing integrated pixel intensity of the amplicons. The methylation index was calculated as M/(M + U) × 100. M, methylated; U, unmethylated. Student’s t test was used to determine statistical significance. H, all primers showed similar efficiencies in discriminating between fully methylated (Methyl) or unmethylated (No Methyl) standard templates (primers for SNCA are shown). *, p < 0.05.
FIGURE 3. Overexpression of α-synuclein results in cytoplasmic retention of Dnmt1 in B103 cells, and this effect might be mediated by the association of both proteins. A, Western blot detection of Dnmt1 in cytoplasmic and nuclear extracts from B103 cells infected with LV-control or LV-α-syn. B, blocking assays to determine the specificity of the −183-kDa band detected with Dnmt1 antibody. C, immunohistochemical analysis of Dnmt1 in B103 cells (arrows, nuclear; stars, cytoplasmic). D, detection of nuclear Dnmt1 (arrows) in B103 cells infected with LV-β-syn. Bars in C and D represent 10 μm. E and F, image analysis showing the percentage of Dnmt1 immunoreactive nuclei. G and H, immuno precipitation (IP) assays showed association of Dnmt1 with α-syn in lentivirus-infected 293T cells (G) and in postmortem human cortical samples (H). WB, Western blot. I, negative immunoprecipitates were obtained when anti-Dnmt1 or anti-α-syn was used to immunoprecipitate homogenates from 293T cells co-infected with LV-Dnmt1 and LV-α-syn. J, detection of Dnmt1, α-syn, and 5-mCi n in B103 cells infected with the indicated lentivirus (arrows, nuclear; arrowheads, cytoplasmic). Bars represent 5 μm. K, image analysis showing the percentage of Dnmt1 immunoreactive nuclei. L, image analysis showing the percentage of 5-mCi immunoreactivity in the nucleus. Student's t test was used to determine statistical significance, * or #, p < 0.05.
previous findings, neither anti-Dnmt1 nor anti-β-syn antibodies were able to pull down the opposite proteins from 293T cells co-infected with LV-Dnmt1 and LV-β-syn, suggesting a lack of association between Dnmt1 and β-syn (Fig. 3f).

Cytoplasmic Retention of Dnmt1 Can Be Rescued by Lentiviral Delivery—We next evaluated whether overexpression of Dnmt1 might revert its cytoplasmic retention in the context of high levels of α-syn. We infected B103 neuronal cells with LV-control, LV-α-syn, LV-Dnmt1 Myc, or a combination of them. Cells were analyzed 48 h after infection, and localization of Dnmt1 was determined by immunocytochemistry. As seen previously, cells infected with either LV-control or LV-Dnmt1 Myc showed nuclear localization of Dnmt1, whereas cells that received LV-α-syn showed diffuse cytoplasmic staining. Cells co-infected with LV-α-syn and LV-Dnmt1 Myc, on the other hand, showed partial restoration of the nuclear localization of Dnmt1-Myc signal, suggesting that a fraction of the newly expressed protein is indeed shuttled into the nucleus (Fig. 3, j and k). To assess whether recovery of nuclear localization of Dnmt1 also rescues the deficiency in DNA methylation, we compared 5-3H methylation between the different groups (Fig. 3, j and l). A significant decay in 5-3H-labeled DNA associated with cytoplasmic retention of Dnmt1 in LV-α-syn-infected cells. Cells that were co-infected with LV-α-syn and LV-Dnmt1 showed partial recovery of 5-3H-labeled DNA that was similar to those of control cells, suggesting that the Dnmt1 molecules that reached the nucleus actively methylated the DNA.

In addition, we investigated whether the dramatic changes in Dnmt1 nuclear content were also present in a transgenic mouse model of PD/DLB expressing wild type human α-syn (α-syn tg) (11). In agreement with our findings in the human brain, significant decays in Dnmt1 protein were detected in α-syn tg brain samples, accompanied by cytoplasmic retention of Dnmt1, global DNA hypomethylation, and association of Dnmt1 with α-syn (supplemental Fig. 1, A–I). These results suggest the adequacy of the transgenic model to investigate this novel epigenetic feature of α-synucleinopathies. Next, we tested whether rescue of Dnmt1 cytoplasmic retention can be achieved in vivo by intracerebral delivery of LV-Dnmt1 in the tg mice (supplemental Fig. 2). Once more, non-transgenic animals showed nuclear localization of Dnmt1 in the frontal cortex, whereas α-syn tg mice presented a diffuse cytoplasmic staining for Dnmt1. In contrast, brains from tg animals that received LV-Dnmt1 injections showed Dnmt1 signal localized to both the cytoplasmic and the nuclear compartments, suggesting that lentiviral injection of Dnmt1 was effective in partially restoring its nuclear localization.

**DISCUSSION**

In the present study, we report a decrease in DNA methylation in the regulatory regions of specific genes in PD and DLB brains. This altered epigenetic regulation is related to mislocalization of Dnmt1, and lentivirus-driven expression of Dnmt1 rescues this defect in neuronal and transgenic mouse models of PD/DLB.

DNA methylation regulates genome reprogramming, cell differentiation, and gene expression. Involvement of aberrant DNA methylation in PD pathology has been suggested by two recent studies showing hypomethylation of a CpG-rich region at intron 1 of the SNCA gene (9, 10). Therefore, we investigated the mechanisms underlying altered DNA methylation in Lewy body diseases, including PD and DLB. We present evidence of reduction of nuclear Dnmt1 in human postmortem cortical brain samples from PD and DLB patients and in the anterior brain region of transgenic mice models expressing human α-syn. Moreover, we found that Dnmt1 is sequestered in the cytoplasm and associated with α-syn.

Alterations in Dnmt1 levels might directly impact on DNA methylation, and in agreement with our previous findings, we indeed detected a significant reduction in global DNA methylation in the brains of PD and DLB patients and of α-syn transgenic mice. Moreover, we present evidence for a decay of methylation at specific CpG islands of SNCA, SEPW1, and PRKAR2A in PD and DLB brains. Although the contribution of the later two genes to pathology remains to be clarified, the expression of both was reported to be up-regulated in PD (17), in line with the notion that DNA methylation is a transcriptional repressor.

Although epigenetic remodeling of chromatin was believed to be mostly restricted to actively dividing cells, DNA methylation has recently been suggested to represent a dynamic event in postmitotic neurons as well, where it has important roles in learning and memory (16). Persistent, gene-specific cortical hypermethylation is induced in rats by a single hippocampus-dependent associative learning experience (19). Moreover, Dnmt expression is up-regulated in the adult rat hippocampus after contextual fear conditioning and is associated with the rapid methylation and transcriptional silencing of the memory suppressor gene Ppi7 (7). Thus, dynamic changes in DNA methylation enable the brain to respond to experience and to stabilize remote memory in the frontal cortex, a process that would require the continuous maintenance of methylation. Furthermore, another set of studies has reported a significant increase in DNA methylation at CpG islands of genes involved in DNA binding and regulation of transcription with age, in support of a role for DNA methylation in the human cerebral cortex throughout the lifespan, which has a broad impact on gene expression in the adult brain (20–22).

In the present study, we report that Dnmt1 associates with α-syn not only in B103 neuronal cells overexpressing the proteins but also in brain tissue from postmortem frontal cortex samples of control subjects and PD and DLB patients, suggesting that this interaction holds true for the endogenous proteins. Association of Dnmt1 with α-syn appears to be a specific event because cytoplasmic retention was not induced by overexpression of the related protein β-syn, and Dnmt1 was not immunoprecipitated by anti-β-syn antibody in protein homogenates. Interestingly, a distinctive feature between α- and β-synucleins is the ability of α-syn to aggregate and accumulate in Lewy bodies. We observed an inverse relation between α-syn oligomerization and Dnmt1 nuclear content that might indicate that sequestration of Dnmt1 is increased by α-syn aggregation. Because Dnmt1 was detected in immunoprecipitates from PD and DLB cases as well as in control brains, interaction with monomeric α-syn is also plausible. We postulate that overexpression and aggregation of α-syn over time during the long
course of PD and DLB pathologies could lead to aberrant association and sequestration of Dnmt1 in the cytoplasm. Further experiments will be needed to determine the causes of cytoplasmic retention of Dnmt1 in PD and DLB, and we cannot exclude the possibility that other mechanisms, such as increased degradation of Dnmt1 in the nucleus or deterioration of nuclear pore complexes in aged cells, might also be involved in this abnormal subcellular localization.

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