Brief Communications

Methylation Regulates Alpha-Synuclein Expression and Is Decreased in Parkinson's Disease Patients' Brains

Ahmad Jowaed,* Ina Schmitt,* Oliver Kaut, and Ullrich Wüllner
Department of Neurology, Rheinische Friedrich-Wilhelms-Universität, Universitätsklinikum Bonn, D-53105 Bonn, Germany

Alpha-synuclein (*SNCA*) is a major risk gene for Parkinson's disease (PD), and increased *SNCA* gene dosage results in a parkinsonian syndrome in affected families. We found that methylation of human *SNCA* intron 1 decreased gene expression, while inhibition of DNA methylation activated SNCA expression. Methylation of *SNCA* intron 1 was reduced in DNA from sporadic PD patients' substantia nigra, putamen, and cortex, pointing toward a yet unappreciated epigenetic regulation of *SNCA* expression in PD.

Introduction

Alpha-synuclein (SNCA) is a key component of Lewy bodies found in Parkinson's disease (PD) patients (Spillantini et al., 1997; Braak et al., 2003). Point mutations and multiplications of *SNCA* cause familial parkinsonian syndromes with high penetrance (Singleton et al., 2003). Several studies suggest that the *SNCA* gene harbors significant risk haplotypes for sporadic PD too (Mizuta et al., 2006). Comprehensive promoter analyses and recent studies of *SNCA* mRNA levels in dopaminergic neurons strengthened the hypothesis that genotype-dependent regulatory mechanisms and increased expression of *SNCA* could contribute to the risk of sporadic PD (Maraganore et al., 2006; Gründemann et al., 2008).

Beyond the actual code of a regulatory DNA sequence, several additional levels of epigenetic transcriptional control have become apparent recently (Suzuki and Bird, 2008). Methylation of CpG dinucleotides is a prime epigenetic mechanism and a frequent biochemical modification of DNA in the human genome. Hypermethylation of CpG-rich regions [or CpG "islands" (CGIs)] is often found in regulatory 5′ regions, and methylation-dependent silencing of tumor suppressor genes is a widely acknowledged mechanism in the pathogenesis of cancer (Feinberg, 2007; Suzuki and Bird, 2008). Aberrant DNA methylation has also been associated with psychiatric conditions and might constitute a pathogenic mechanism for other diseases as well (Feinberg, 2007).

We investigated whether aberrant methylation might contribute to a presumed dysregulation of SNCA expression in sporadic PD.

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*A.J. and I.S. contributed equally to this work.

Correspondence should be addressed to Ullrich Wüllner, Department of Neurology, Universitätsklinikum Bonn, Sigmund-Freud-Strasse 25, D 53105 Bonn, Germany. E-mail: wuellner@uni-bonn.de.

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Materials and Methods

WEBGENE was used for the prediction of CGIs, and the TESS (Transcription Element Search Software, http://www.cbil.upenn.edu/tess) program for identification of putative transcription factor (TF) binding sites and proscan (version 1.7, http://www-bimas.cit.nih.gov/molbio/proscan/) were used for prediction of putative promoter regions.

SK-N-SH cells were cultured in RPMI 1640 (PAA) supplemented with 10% fetal bovine serum (FBS Gold, PAA), 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA) at 37°C and 5% CO_2. For treatment with 5-aza-2′ deoxycytidine (Aza; A3656, Sigma), 5×10^6 cells were seeded in 10 cm plates, cultured overnight, and treated with 10 μM Aza (dissolved in DMSO) or with DMSO as control. The treatment was repeated (including medium replacement) every 8 h for 24 or 48 h. Cells were harvested into three portions for isolation of DNA, RNA, and proteins.

DNA isolation and bisulfate treatment was performed as previously described (Pieper et al., 2008). Bisulfite-treated DNA was amplified using SYN-PromF AAAATTTTGAAGATATTTGAATTAAAG/SYN-PromR-CTAATCCTCCTCCTTCTCCTTCTC and SYN-IntF-GGAGTTTAAGGAA-AGAGATTTGATT/SYN-IntR-CAAACAACAAACCCAAATATAATAA, specifically designed for the bisulfite-treated DNA. PCR products ($SNCA_{(-2079/-1507)}$ and $SNCA_{(-926/-483)}$) (Fig. 1*b*) were cloned into pCR 2.1 TOPO vector (Invitrogen) following the manufacturer's instructions. Plasmid DNA was isolated from at least 10 clones per region (Wizard Plus SV Minipreps, Promega), sequenced using vector-specific primers and the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Quality control for DNA methylation data was performed using BiQ (software tool for DNA methylation analysis; http://biq-analyzer.bioinf.mpi-inf.mpg.de/).

RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. For reverse transcription, 1 μg of total RNA and m-MLV Reverse Transcriptase, RNase H Minus (Promega) was used with oligo dT and random hexamer primers according to the manufacturer's instruction. Quantitative PCR was performed in triplicate with SYBR Green JumpStart Taq ReadyMix (Sigma) and SNCA-primers (SNCA-F_GGACCAGTTGGGCAAGAATG and SNCA-R_GGGCACATTGGA-ACTGAGCAC) on an Applied Biosystems 7500 Fast Real-Time PCR System using 2 μl of 1:10 diluted cDNA for each reaction.

Protein extracts were prepared by resuspending the cells in lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF, 1× protein inhibitor mix Complete Mini (Roche Diagnostics). After centrifugation, 50 μg of protein per lane was separated by SDS-PAGE, and detection was performed with the ECL Western blotting detection system (GE Healthcare)

using secondary antibodies conjugated with horseradish peroxidase. We used antibodies at the following dilutions: anti-Synuclein (1:2000; catalog #610786, BD Bioscience) and goat anti-mouse (1:2000; catalog #115-035-003, Jackson ImmunoResearch).

Genomic DNA from control lymphocytes was used as a template for PCR (LYMF-GAGAAGGAGGAGGACTAGGAGG and LYMR-AGCATCTCCCATCTTGG), and the PCR product was inserted into pGL4.23(luc/min P) vector (Promega). The resulting construct ($SNCA_{(-1524/-189)}$) expressed the *Firefly* luciferase under the control of the *SNCA* exon 1/intron 1 fragment (Fig. 1*c*). The reverse insertion of the fragment into pGL4.23(luc/min P) served as a control. The luciferase experiment was performed in triplicate and repeated at least four times.

For *in vitro* methylation, 15 μg of the reporter constructs was incubated with 32 U of SssI (CpG) methylase (New England Biolabs), supplemented with 32 mM S-adenosylmethionine (SAM, New England Biolabs), and incubated overnight at 37°C. The reaction was purified using Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions.

HeLa cells were cultured as SK-N-SH cells (see above). A total of 1×10^6 cells per well were seeded on 24 well plates and transfected with the indicated luciferase reporter constructs with/without *in vitro* methylation using Lipofectamine 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. *Renilla* luciferase (pRL-CMV, Promega) was cotransfected to normalize for transfection efficiency. Cells were harvested 24 h after transfection and luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) in a Centro LB 960 luminometer (Berthold Technologies).

DNA from substantia nigra pars compacta (SNpc) and cortex from six PD patients (three males, three females; mean age, 79.3 ± 5.6 years) and six neurologically healthy control individuals (four males, two females; mean age, 75.8 ± 6.2 years) (provided by the GermanBrainNet), and DNA from putamen from an additional 14 individuals (PD: 3 males, 3 females; mean age 77.0 ± 5.7 years; controls: 2 males, 6 females; mean age 78.0 ± 4.1 years) (provided by Prof. P. Riederer, University of Würzburg, Würzburg, Germany) was used for analysis. The reported concomitant diseases and the causes of death revealed no systematic differences between the control group and the PD patients (comprehensive clinical information is provided in supplemental Table 1, available at www.jneurosci.org as supplemental material). DNA was extracted from frozen tissue using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. Bisulfite sequencing of $SNCA_{(-926/-483)}$ was performed as described above. Plasmid DNA was isolated from at least 10 clones per individual per region. Independent replication experiments were performed on cortex-derived DNA from an additional 10 clones per individual.

Statistical analysis was performed using one-way ANOVA.

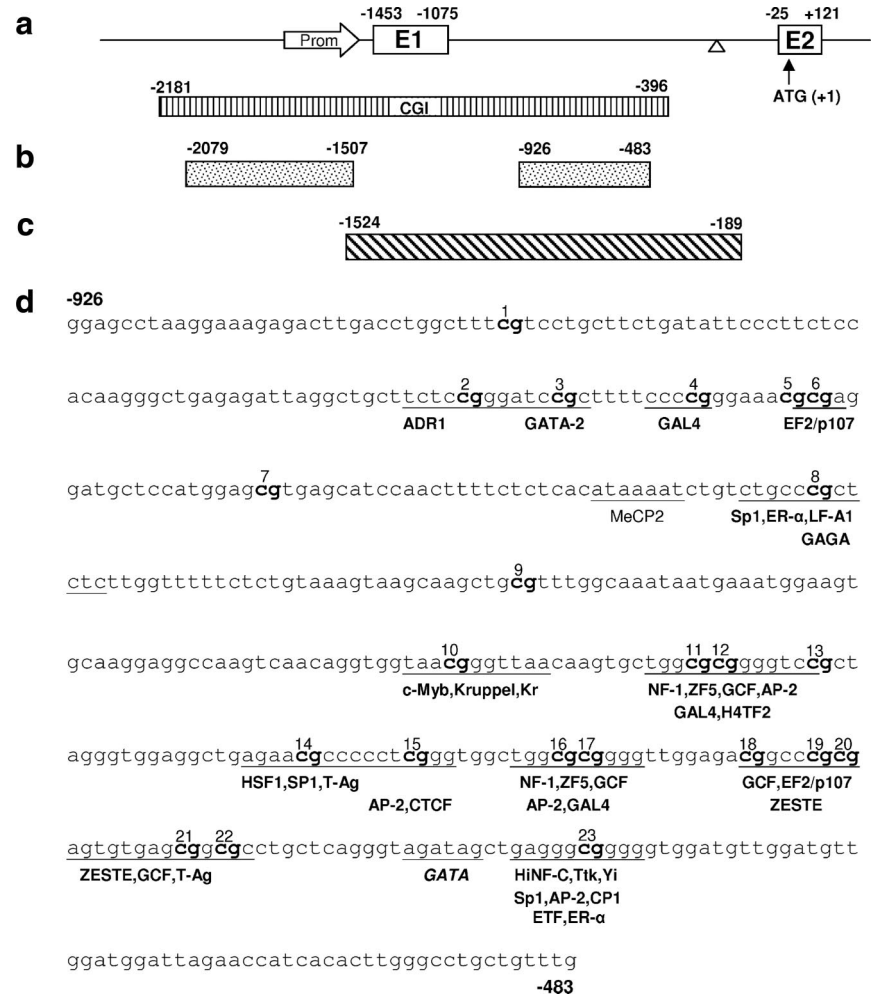

Figure 1. The *SNCA* region of interest. ***a***, Schematic drawing of the 5′ region of *SNCA* with exons 1 and 2 (boxes) and the 5′ UTR and intron 1 (line). The arrow indicates the position of the putative core promoter; the triangle indicates the position of a rudimentary TATA box. Dimension of the CGI is depicted by the stripped box. Numbers are relative to the translation start site (ATG + 1). ***b***, Fragments $SNCA_{(-2079/-1507)}$ (promoter) and $SNCA_{(-926/-483)}$ (intron) were used for bisulfite sequencing. ***c***, Fragment $SNCA_{(-1524/-189)}$ was used for reporter assays. ***d***, Predicted transcription factor binding sites within $SNCA_{(-926/-483)}$ (intron) adjacent to CpG dinucleotides.

Results

Methylation status of *SNCA* intron 1 affects *SNCA* expression

The human *SNCA* gene contains a CpG island extending from −2181 to −396 upstream from the translation start site (Fig. 1*a*), enclosing the canonical promoter and a putative concealed promoter in intron 1 (−1074 to −26), which has been linked to NGF/basic FGF-mediated *Snca* expression in rodents (Xia et al., 2001; Clough and Stefanis, 2007). Further supporting the significance of intron 1 in *Snca* expression, GATA TF binding in intron 1 has been shown to regulate *Snca* transcription (Scherzer et al., 2008).

We therefore analyzed the methylation state of *SNCA* in a promoter region fragment and in an intronic sequence stretch of SK-N-SH cells. Bisulfite sequencing of $SNCA_{(-2079/-1507)}$ (core promoter) and $SNCA_{(-926/-483)}$ (intron 1) (Fig. 1*b*), revealed that only 1.4 ± 1.5% of the 40 CpG sites in $SNCA_{(-2079/-1507)}$ were methylated, in contrast to 70 ± 20.3% of the 23 CpG sites in $SNCA_{(-936/-483)}$ (Fig. 2*a*,*b*; supplemental Table 2, available at www.jneurosci.org as supplemental material). To investigate the functional significance of intron 1 methylation, we used the U.S. Food and Drug Administration-approved DNA methylation inhibitor Aza. Aza treatment for 24 and 48 h reduced the methylation state of $SNCA_{(-926/-483)}$, while no effect on the marginal methylation of $SNCA_{(-2079/-1507)}$ became apparent (Fig. 2*a*,*b*). The Aza-induced decreased methylation of $SNCA_{(-926/-483)}$ resulted in increased amounts of *SNCA* mRNA and increased SNCA protein expression (Fig. 2*c*,*d*).

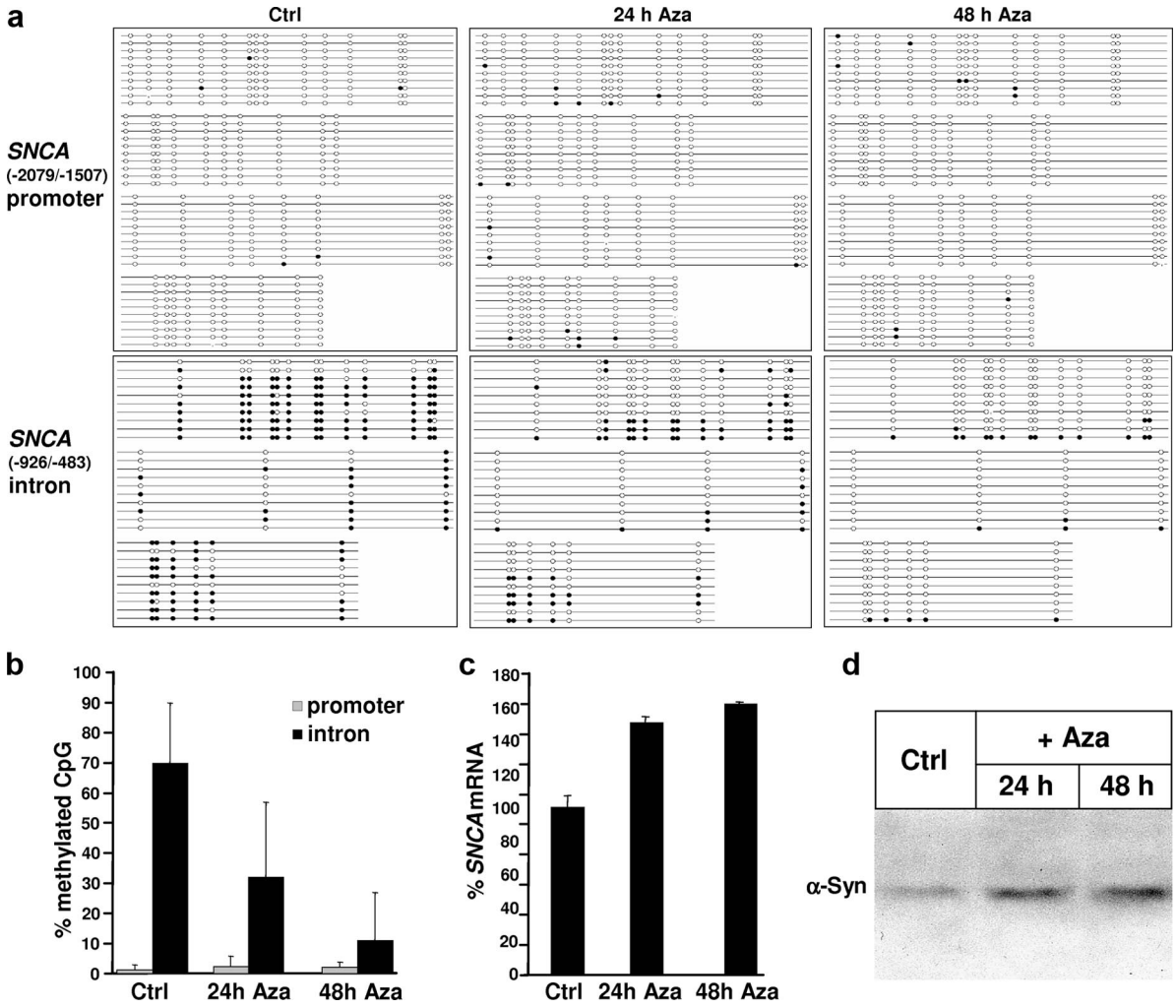

Figure 2. Status of *SNCA* methylation in SK-N-SH cells and impact on expression. ***a***, SK-N-SH cells were incubated with DMSO [control (Ctrl)] or 10 μM Aza for 24 and 48 h, respectively, and isolated DNA was used for bisulfite sequencing of $SNCA_{(-2079/-1507)}$ (promoter) and $SNCA_{(-926/-483)}$ (intron); 10 independent clones for each condition were analyzed by bisulfite sequencing. The lollipop diagram with unmethylated CpG sites (open circles) and methylated CpG sites (closed circles) illustrates the higher degree of methylation in $SNCA_{(-926/-483)}$ (intron) compared with $SNCA_{(-2079/-1507)}$ (promoter) and the decrease of methylated CpG in $SNCA_{(-926/-483)}$ (intron) after addition of Aza. ***b***, After 48 h of Aza treatment, the fraction of methylated CpGs is reduced by ~60% in $SNCA_{(-926/-483)}$ (intron). ***c***, Treatment with Aza induced a time-dependent increase of *SNCA* expression exceeding 50% of Ctrl. Quantitative RT-PCR was performed in triplicate and presented as the mean ± SD. ***d***, Representative Western blot with anti-synuclein antibody demonstrates an increase of synuclein protein after addition of Aza too.

Promoter activity of intron 1 is influenced by methylation

Next, we analyzed the impact of methylation on gene expression activity in luciferase reporter experiments. $SNCA_{(-1530/-193)}$, a reporter construct chosen to comprise intron 1 (containing 71 CpG dinucleotides), displayed significant promoter activity (>30-fold higher activity compared with the construct with the inverted insert $SNCA_{(-193/-1530)}$, which served as control) (Fig. 3). After treatment with SssI CpG-methyl transferase, which selectively adds a methyl group to all cytosine residues within CpG dinucleotides, the activity of the methylated construct was decreased to control levels (Fig. 3). When SssI or SAM as the methyl group donor was omitted, the promoter activity was not altered significantly.

Methylation of *SNCA* intron 1 is reduced in DNA from PD patients

To investigate whether epigenetic changes might contribute to a presumed dysregulation of SNCA expression in sporadic PD, we analyzed DNA from three different regions (SNpc, putamen, and cortex) in 37 tissue samples from 12 PD patients and 14 controls. We found significantly fewer methylated CpG sites in PD patients' DNA (Fig. 4*a*; supplemental Table 3, available at www.jneurosci.org as supplemental material). The mean methylation rate of $SNCA_{(-926/-483)}$ (intron 1) in human brain was 10-fold lower compared with SK-N-SH cells (7 vs 70%), yet, similar to SK-N-SH cells, $SNCA_{(-2079/-1507)}$ (promoter) displayed only sparse methylation (~1.5%; data not shown). The *SNCA* intron 1 methylation pattern varied considerably between tissues and individuals (Fig. 4*b*). Despite this variability, differences between PD and control were found not only in the group comparison (Fig. 4*a*) but also in SNpc and putamen DNA at specific positions (8, 12, and 17) within intron 1, located within predicted consensus binding sites of TFs (Figs. 1*d*, 4*b*). Except for positions 1, 5, 7, and 9, all CpG sites were located within predicted TF binding sites (Fig. 1*d*), suggesting that *SNCA* intron 1-mediated transcriptional regulation may depend on the binding of specific TFs.

Discussion

We identified $SNCA_{(-1524/-189)}$ in intron 1 as a methylation-dependent, transcriptionally active region of the human *SNCA*

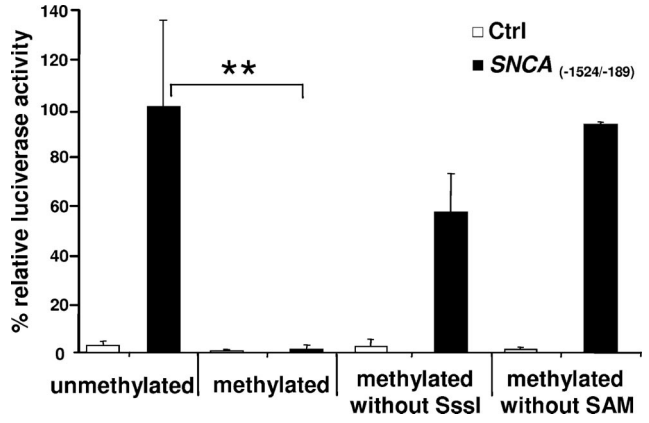

Figure 3. Promoter activity of $SNCA_{(-1524/-189)}$ in HeLa cells. *In vitro* methylation of the reporter construct $SNCA_{(-1524/-189)}$ reduced relative luciferase activity by 98.5% (**$p \leq 0.01$, one-way ANOVA). Absence of SssI or SAM from the *in vitro* methylation reaction minimized reduction. Ctrl, Construct with the inverted insert.

gene and found that $SNCA_{(-926/-483)}$ is hypomethylated in sporadic PD patients' brains. A yet unappreciated epigenetic control of *SNCA* expression might contribute to the presumed dysregulation of *SNCA* expression in PD.

The finding that the methylation of intron 1 modulates human *SNCA* transcriptional activity expands recent studies, which independently identified expression-relevant GATA binding sites and NGF response elements, respectively, in intron 1 of rodent *Snca* (Clough and Stefanis, 2007; Scherzer et al., 2008). In addition, promoter prediction software identified a rudimentary TATA box and several TF binding sites immediately upstream from the ATG (+1) in human *SNCA* intron 1 (Fig. 1*a*,*d*). In contrast to exon 1/intron 1 of the rat *Snca* gene, which harbors only 19 CpG sites, the human *SNCA* exon 1/intron 1 carries 66 CpG sites; thus, changes in *SNCA* methylation may have more pronounced regulatory effects in humans compared with rodents. The possibility that intron 1 serves to generate a relevant fraction of *SNCA* transcripts is further supported by the original work by Uéda et al. (1993), who identified a principal *SNCA* transcript in human brain ~1.500 bp in size, which corresponds well to the size of exons 2 to 6 (1.522 bp). Inclusion of the canonical exon 1, though, would extend the predicted mRNA length up to 1.902 bp. Thus, the canonical exon 1 and intron 1 may serve regulatory, perhaps methylation-sensitive, functions.

This is particularly intriguing in the light of the second principal finding, i.e., that intron 1 $SNCA_{(-926/-483)}$ was found to be hypomethylated in sporadic PD patients' brains. Hypomethylation was consistently observed across the analyzed CpG sites in all three brain regions investigated, although the *SNCA* methylation patterns varied considerably between tissues and individuals. Regional variation of methylation within the brain has also been found previously for the tumor necrosis factor-α promoter (Pieper et al., 2008), and tissue-specific variation in DNA methylation levels was found along human chromosome 1 (De Bustos et al., 2009).

The differentially methylated CpG sites are associated with predicted TF binding sites, suggesting that reduced methylation could promote SNCA expression in PD brain. We propose that less methylated *SNCA* is more likely to be actively transcribed in response to particular stimuli. Whether *SNCA* mRNA levels are indeed increased in PD patients' brains has been addressed in several previous studies, and although some studies failed to detect differences in *SNCA* mRNA levels, others indicated that at least SNpc tissue in PD contained more *SNCA* mRNA (Chiba-Falek et al., 2006). This was confirmed in a recent study using laser dissecting microscopy of PD SNpc neurons (Gründemann et al., 2008).

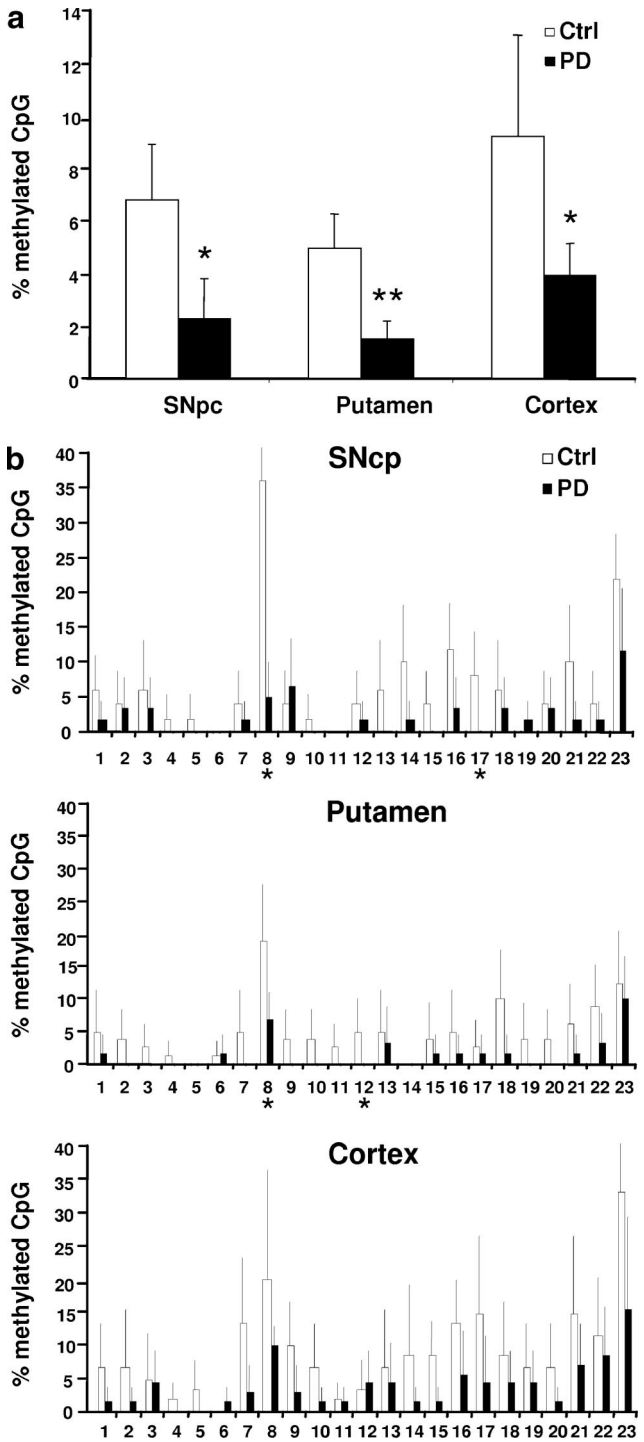

Figure 4. Status of $SNCA_{(-926/-483)}$ methylation in human brain. ***a***, Mean percentages (±SD) of methylated CpG sites summed up over all 23 CpG sites analyzed. PD patients' DNA was hypomethylated compared with the neurologically healthy individuals in all analyzed brain regions. *$p \leq 0.05$, **$p \leq 0.01$. ***b***, Detailed comparison of the methylation of $SNCA_{(-926/-483)}$ in DNA from SNpc, putamen, and cortex of PD and neurologically healthy individuals. Percentage of methylated CpG at the particular position in the three different brain regions. Asterisks indicate significant ($p \leq 0.05$, one-way ANOVA) hypomethylation in PD patients compared with control (mean ± SD of at least 10 clones per individual per CpG position analyzed by bisulfite sequencing).

DNA methylation turns out to be a highly dynamic process (Feinberg, 2007). Major age-dependent changes of gene methylation have been reported not only in neonatal development but also in the adult CNS, where distinct age-dependent changes became apparent in subsets of cortical neurons (Siegmund et al., 2007). In addition to classic monogenetic epigenetic disease, i.e., Beckwith–Wiedeman and Prader–Willi syndromes, recent data point to an epigenetic component also of other neuropsychiatric disorders, notably in Rett syndrome (Chahrour and Zoghbi, 2007). The decreased methylation of *SNCA* intron $1_{(-926/-483)}$ could contribute to increased expression of SNCA in PD patients' brains, as $SNCA_{(-1524/-189)}$ acted as a methylation-dependent, hitherto unappreciated, transcriptionally active region of the human *SNCA* gene.

At present, we can only speculate about the cause of this hypomethylation. An inherited damage of the methylation machinery appears rather unlikely in the light of recent whole-genome association studies that identified only SNCA and the MAPT regions as major denominators of PD risk (Simón-Sánchez et al., 2009; Edwards et al., 2010). Consequently, in the adult PD brain we did not find an overt loss of the maintenance methylase DNMT1 (Kaut et al., 2009). Whether a disturbance of the *de novo* methyltransferases DNMT 3a and 3b early in the development of the CNS could contribute to the observed changes remains unknown.

One might presume that methylation could be affected by environmental factors. Nutrition, i.e., famine, has profound effects on DNA methylation, and deficiency in folate and methionine, which are necessary for the biosynthesis of methyl donors for methylcytosin, can lead to aberrant imprinting of IGF2 (Waterland et al., 2006). Although we have shown previously that a methylenetetrahydrofolate reductase (*MTHFR*) genotype, which affects methionine 1-carbon metabolism, modulates age of onset in PD (Wüllner et al., 2005), we found no difference in the methylation of the imprinted *IGF2* gene in PD (Kaut et al., unpublished observations). Other lines of evidence suggest that critical psychosocial events (e.g., early life stress) and environmental exposures may induce lasting epigenetic marks (Christensen et al., 2009; Murgatroyd et al., 2009). The methylation state of *SNCA* intron 1 might constitute such a mark, adding an additional level of complexity to a graded (epi-)genetic risk for PD.

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