Published in final edited form as:

Mov Disord. 2014 November ; 29(13): 1606–1614. doi:10.1002/mds.25784.

DNA Methylation of *MAPT* Gene in Parkinson's Disease Cohorts and Modulation by Vitamin E *In Vitro*

Kirsten G. Coupland, BSc^{1,2}, Goerge D. Mellick, PhD³, Peter A. Silburn, PhD, FRACP⁴, Karen Mather, PhD⁵, Nicola J. Armstrong, PhD^{5,6,7}, Perminder S. Sachdev, MD^{5,8}, Henry Brodaty, DSc^{5,9}, Yue Huang, MD^{1,2}, Glenda M. Halliday, PhD^{1,2}, Marianne Hallupp, BSc¹, Woojin S. Kim, PhD^{1,2}, Carol Dobson-Stone, DPhil^{1,2,#}, and John B.J. Kwok, PhD^{1,2,#}

¹Neuroscience Research Australia, Sydney, NSW, Australia

²School of Medical Sciences, University of New South Wales, Sydney, NSW, Australia

³Eskitis Institute for Cell and Molecular Therapies, Griffith University, Brisbane, Qld, Australia

⁴University of Queensland Centre for Clinical Research, Herston, QLD, Australia

⁵Centre for Healthy Brain Aging, School of Psychiatry, University of New South Wales, Sydney, NSW, Australia

⁶School of Mathematics and Statistics, University of Sydney, NSW, Australia

⁷School of Mathematics and Statistics and Prince of Wales Clinical School. University of New South Wales, Sydney, NSW, Australia

⁸Neuropsychiatric Institute (NPI), Prince of Wales Hospital, Sydney, NSW, Australia

⁹Aged Care Psychiatry, Prince of Wales Hospital, Sydney, NSW, Australia

Abstract

Background—Parkinson's disease (PD) is a neurodegenerative disorder for which environmental factors influence disease risk and may act via an epigenetic mechanism. The microtubule-associated protein tau (*MAPT*) is a susceptibility gene for idiopathic PD.

Methods—Methylation levels were determined by pyrosequencing of bisulfite treated DNA in a leukocyte cohort (358 PD and 1084 controls) and two brain cohorts (Brain1 comprising 69 cerebellum controls, Brain2 comprising 3 brain regions from 28 PD and 12 controls). *In vitro* assays involved the transfection of methylated promoter-luciferase constructs or treatment with an exogenous micronutrient.

Correspondence to: John B.J. Kwok.

#Joint senior authors

Supplemental data: Supplemental Appendix (electronic file name: Couplandetal_Supplementary_e1_R1)

Author contributions: JBJK and CDS conceived the study. KGC, KM, NJA, WSK, MH, YH performed experiments. KGC, JBJK and CDS analysed the data and drafted the manuscript. HB, PS, KM, GDM, PAS and GMH organized the human tissue study (tissue collection and diagnosis, ethics and tissue request). GMH is CI on the research programs that longitudinally follow brain donors with Parkinson's disease and she is also director of the Sydney Brain Bank.

Results—In normal leukocytes, *MAPT*H1/H2 diplotype and gender were predictors of *MAPT* methylation. Haplotype-specific pyrosequencing confirmed H1 haplotype to have higher methylation than H2 in normal leukocyte and brain tissues. *MAPT* methylation was negatively associated with *MAPT* expression in Brain1 cohort and transfected cells. Methylation levels differed between three normal brain regions (Brain2, putamen > cerebellum > anterior cingulate cortex). In PD samples, age at onset was positively associated with *MAPT* methylation in leukocytes. Moreover, there was hypermethylation in the cerebellum and hypomethylation in the putamen of PD patients compared with controls (Brain2 cohort). Finally, leukocyte methylation status was positively associated with blood Vitamin E levels, the effect being more significant in H2 haplotype carriers; this result was confirmed in cells exposed to 100 mM Vitamin E.

Conclusions—The significant effects of gender, diplotype and brain region suggest that hypermethylation of the *MAPT* is neuroprotective by reducing *MAPT* expression. Vitamin E effect on *MAPT* represents a possible gene-environment interaction.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by tremor and bradykinesia that affects 2% of the population over the age of 65.¹ The neuropathologic hallmarks for PD are a loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies, cytoplasmic inclusions composed of α -synuclein.² Only 4% of PD cases are familial forms linked to mutations in single genes while the remaining are 'idiopathic', as their aetiology is unknown.³ Genome-wide association studies have implicated the gene encoding microtubule-associated protein tau (*MAPT*), a protein that aids in stabilising the axonal cytoskeleton, as a major susceptibility locus for idiopathic PD.^{4,5} There are two main *MAPT* haplotypes, termed H1 and H2, resulting from single nucleotide polymorphisms (SNPs) in absolute linkage disequilibrium that spans the entire *MAPT* gene.⁶ The *MAPT* haplotypes have been demonstrated to affect expression and/or alternate splicing using *in vitro* promoter assays and *in vivo* measurements of transcripts in brain samples.⁷⁻¹⁰ Thus, other mechanisms that alter the expression of *MAPT* could be pathogenic pathways in PD.

Heritable changes in gene expression that do not involve coding sequence modifications are referred to as 'epigenetic'. One form of epigenetic modification, DNA methylation, involves the reversible attachment of a methyl group to a cytosine/guanine (CpG) dinucleotide.¹¹ Aberrant DNA methylation is well documented in cancer¹², and now an area of interest in neurodegenerative conditions.^{13,14} Jowaed et al identified differential methylation in PD patients at intron 1 of the gene encoding α -synuclein (*SNCA*), another susceptibility locus for late-onset PD.¹⁵ Examination of the same CpG island in post-mortem PD brains revealed that the substantia nigra exhibited lower amount of methylation than controls.¹⁶ A study investigating methylation of *MAPT* examined brain tissue of non-neurodegenerative subjects and found that overall methylation decreased with age, but increased at binding sites for transcription factor Sp1.¹⁷ A study comprising post-mortem brain tissue from 124 individuals with different forms of tauopathies failed to find any significant differences in methylation state of the *MAPT* gene as measured by mass spectrometry,¹⁸ although data with this technique is difficult to interpret in the presence of polymorphisms.

We postulate that an additional aspect of the pathogenic mechanism associated with *MAPT* could be a diplotype-specific methylation of the *MAPT* promoter. In this study, we examined the methylation state of the promoter region of *MAPT* by pyrosequencing in leukocyte and brain DNA cohorts and identified patterns of methylation associated with diplotype, gender, brain region, blood micronutrient levels, age-at-onset of PD, disease status, and *MAPT* expression.

Materials and Methods

A detailed description of materials and methods are included in the supplementary material.

Study cohorts

To examine the effects of major demographic, genetic and disease-related changes in *MAPT* methylation, three cohorts were examined comprising a total of 1442 leukocyte DNA samples^{19,20} and 109 patient brain tissue DNA samples (table 1). Leukocyte cohort was used to determine the effects of the co-variables of age, gender and *MAPTH1/H2* diplotypes in predicting the level of *MAPT* methylation in normal and PD cases. Brain1 cohort was used to determine the downstream effects of *MAPT* methylation on gene expression. Three brain regions differing in their PD pathology were examined in Brain2 cohort to assess region and disease-specific effects.²¹ Informed consent was obtained from all participants, and the project was approved by the relevant institutional ethics committee.

DNA extraction and genotyping

Taqman Probe Genotyping Assays (ABI Biosystems, CA, USA) for rs76594404 were used to determine the corresponding H1/H2 *MAPT* diplotype in the Brain1 and 2 cohort. rs242559, previously genotyped as part of the Affymetrix SNP 6.0 array (CA, USA), was used to determine the corresponding H1/H2 diplotypes in the Leukocyte cohort.

Pyrosequencing

DNA samples were bisulfite converted using the EpiTect 96 Bisulfite Kit (QIAGEN, Venlo, Netherlands) according to manufacturer's specifications. The PyroMark Q24 (QIAGEN) was used to determine methylation a region of the *MAPT* promoter region at the single nucleotide level. Haplotype-specific pyrosequencing²² was based on haplotype-specific sequencing primers, corresponding to either the H1 or H2 allele of the rs76594404 polymorphism (figure 1A). Subcloning of PCR products and Sanger sequencing was used to verify pyrosequencing data.

MAPT promoter methylation assay

MAPT promoter was subcloned into the pCpGL vector (Prof. Dr. Michael Rehli, Germany)²³ and methylated using *SssI* methyltransferase (New England Biosciences, MA, USA). The human neuroblastoma cell line SK-N-F1 (ATCC CRL-2142) were transfected, lysed after 48 h and assayed for luciferase activity using the Dual-Luciferase® Reporter assay system (Promega, WI, USA).

Vitamin E assay

SK-N-F1 cells were incubated for 72 hours in the presence of vitamin E ((+)- α -Tocopherol) (Sigma-Aldrich, NSW, Australia) and methylation of the endogenous *MAPT* gene was determined by pyrosequencing as described previously. Expression of DNMT genes was assayed using Taqman probe technology (Life Technologies, Carlsbad, CA).

Statistical analyses

Independent effect size and *p* values of demographic factors and diplotypes as predictors of leukocyte methylation levels were estimated using multiple linear regression. Age, sex and *MAPT* diplotype were subsequently included as *a priori* covariates in all relevant analyses. Logistic regression was performed to examine the relationship between diplotype, methylation and disease status in Leukocyte cohort. Non-parametric tests were used to assess the effects of *in vitro* (promoter luciferase assays, vitamin E exposure), *ex vivo* (haplotype specific pyrosequencing) analyses, and to compare region-specific methylation in each brain DNA between control and PD samples. All statistical analyses were performed in SPSS v. 20 (IBM, Armonk, NY).

Results

Demographic and *MAPT* diplotype effects on *MAPT* promoter methylation

The *MAPT* gene has a CpG island comprising 302 CpGs spanning the promoter region⁷ and part of intron 1 as defined by the UCSC Genome Bioinformatics Site ([http:// genome.ucsc.edu/](http://genome.ucsc.edu/)) (figure 1A). The methylation levels of a region spanning 6 CpGs within the island were examined by pyrosequencing analysis and correlated with potential confounding factors. A list of all demographic variables, including smoking status, and levodopa medication, is included in supplementary table 1. In normal leukocytes, gender was a significant independent predictor of methylation in leukocyte DNAs with women having significantly higher levels of methylation than men (multiple linear regression; $\beta = 0.17$; $p = 6.4 \times 10^{-10}$; figure 1B; supplementary table 2). Age was not a significant predictor of methylation levels in controls. *MAPT* diplotype was a highly significant independent predictor of methylation in Leukocyte cohort (multiple linear regression; $\beta = 0.40$; $p = 6.0 \times 10^{-44}$; figure 1B; supplementary table 1), with the H1/H1 diplotype predicting 1.5 fold higher levels of methylation than the H2/H2 diplotype (figure 1B). *Ex vivo* analysis of haplotype-specific methylation of $N = 5$ neurologically normal heterozygote individuals selected from each tissue (Leukocyte and Brain2 cohort) confirmed these findings, with significantly higher methylation for the H1 haplotype compared with the H2 haplotype in each individual for all tissue types except anterior cingulate cortex (ACC) (Wilcoxon paired sign rank test; $p = 0.043$ for leukocyte, cerebellum, and putamen; figure 2). Accuracy of the pyrosequencing assay was confirmed by subcloning and Sanger sequencing to determined the mean number of methylated CpG per PCR product, with a significant correlation between mean *MAPT* methylation levels determined by the two assays (linear regression: $r^2 = 0.67$, $p = 3 \times 10^{-5}$, supplementary figure 2).

MAPT methylation and MAPT expression

We examined *MAPT* expression levels, previously quantified by RT-PCR,²⁴ as a function of the gene's methylation in 69 neurologically normal cerebellum DNA samples (Brain1 cohort). Regression analysis including age, sex and *MAPT* diplototype as covariates revealed a significant inverse relationship between *MAPT* methylation and expression (multiple linear regression, $\beta = -0.27$, $p = 0.043$) with a higher level of methylation being associated with a lower level of *MAPT* expression (figure 3A). To determine the effects of methylation *in vitro* we performed a luciferase reporter gene assay. Methylation of the reporter construct led to a significant decrease in luciferase expression for both H1 (Wilcoxon paired sign rank test; 1.3 fold decrease, $p = 0.043$) and H2 (1.4 fold decrease, $p = 0.043$) promoter constructs in SK-N-F1 cells (figure 3B).

MAPT methylation and disease

In PD leukocyte samples, age (multiple linear regression, $b = 0.10$, $p = 0.031$), gender ($b = 0.21$, $p = 9.0 \times 10^{-6}$) and diplotype ($b = 0.43$, $p = 2.8 \times 10^{-18}$) were all significant predictors of methylation (figure 1C; supplementary table 1). Whilst a significant increase in frequency of H1/H1 homozygotes in PD cases compared with controls was observed in the Leukocyte cohort (binary logistic regression; $\text{Exp}(B) = 1.3$, $p = 0.043$), *MAPT* methylation was not a significant predictor of disease status after adjusting for the *a priori* covariates (data not shown, $p > 0.05$). However, *MAPT* methylation was a significant predictor of PD age at onset ($\beta = 0.18$, $p = 0.003$; figure 3C), with higher methylation levels being associated with a later age of onset, after adjusting for the effects of gender and diplotype. For this analysis, age was not included as a covariate as age-at-collection and age-at-onset were highly correlated (linear regression; $\beta = 0.78$, $p = 1.20 \times 10^{-72}$) (supplementary figure 1) and because age-at-collection was not correlated with methylation levels in control leukocytes (table 2). Note that the correlation between *MAPT* methylation and age at onset was still significant (multiple linear regression; $\beta = 0.08$, $p = 0.035$) with the inclusion of age (at collection) as a covariate.

Levodopa prescription information was available for $N = 353$ PD individuals (Leukocyte cohort; mean = 573 mg/day; range = 0 to 2850 mg/day). No dosage information was available for either of the Brain cohorts. Regression analysis revealed a significant effect of levodopa on *MAPT* methylation (multiple linear regression; $\beta = -0.17$, $p = 0.004$) with age, gender and *MAPT* diplotypes as covariates (supplementary figure 3). This is consistent with the known levodopa effect of decreasing S-adenosylmethionine (SAM) levels, leading to DNA hypomethylation.²⁴

Region- and disease-specific differences in methylation levels from the Brain2 cohort were examined after normalization against a mean brain *MAPT* methylation value (obtained from the mean of the three brain regions for each individual) in order to examine the relative trends in methylation within each patient (figure 3D). Post-mortem delay and brain pH were not significantly associated with *MAPT* methylation in any of the three brain regions (data not shown). We observed an overall brain region difference in methylation levels within normal individuals (Friedman's two-way ANOVA; *Friedman's test statistic* = 13.8; $p = 0.001$), with the putamen having the highest levels of methylation, followed by the

cerebellum, and then ACC. We also observed a significant region-specific difference in methylation between control and PD patient samples from the cerebellum and putamen. The cerebellum showed higher levels of normalised methylation in PD brains (Wilson Mann-Whitney U test; $W = 642$, $p = 0.045$) compared with controls. The ACC showed no significant difference between controls and PD ($W = 619$, $p = 0.192$). In contrast, the putamen showed lower normalised methylation in PD subjects ($W = 464$, $p = 7.4 \times 10^{-4}$). To examine whether *MAPT* promoter methylation was varying independently of global DNA methylation levels we used an ELISA assay to examine global methylation levels in 10 control and 10 PD putamen DNA samples from the Brain2 cohort (supplementary figure 4). Regression analysis revealed that global methylation was not a significant predictor of *MAPT* methylation levels (linear regression; $\beta = -0.01$; $p = 0.959$).

Haplotype-specific effects of Vitamin E on *MAPT* methylation

Blood biochemistry data of micronutrients were available for $N = 860$ control individuals from the Leukocyte cohort. A list of all blood biochemistry variables, including lipid levels, is included in supplementary table 1. The levels of vitamin A, vitamin E, beta-carotene, folate and vitamin B12 were examined as predictors of *MAPT* methylation. Vitamin E levels were significantly correlated with *MAPT* methylation levels after adjusting for the co-variates of age, gender and diplotype (multiple linear regression; $\beta = 0.08$, $p = 0.018$; supplementary table 3). Moreover, when stratified into H1 (H1/H1 homozygotes) or H2 carriers (H2 homozygotes and H1/H2 heterozygotes), a stronger effect was observed for vitamin E in H2 carriers ($\beta = 0.19$, $p = 3.8 \times 10^{-4}$; figure 4A and B) compared with H1 homozygotes ($\beta = 0.04$, $p > 0.05$; figure 4A and B). To determine the effects of vitamin E *in vitro*, SK-N-F1 cells were exposed to 0, 50 and 100 mM vitamin E ((+)- α -Tocopherol). SK-N-F1 cells are heterozygous for the H1/H2 haplotype. Haplotype-specific pyrosequencing demonstrated a significant change (Wilcoxon paired sign rank test; 2.1 fold increase; $p = 0.043$) in methylation of the H2 haplotype, but not the H1 haplotype, in cells exposed to 100 mM vitamin compared with untreated cells. Of interest, the H1 haplotype had higher methylation levels than the H2 haplotype in SK-N-F1 cells as well (Wilcoxon paired sign rank test; mean 1.6 fold increase; $p = 0.009$; figure 4C), and is consistent with our observations for leukocytes and brain tissues (figure 2).

DNA methylation is co-ordinated by three DNA methyltransferases isoforms: DNMT1, DNMT3a and DNMT3b.¹³ We examined whether vitamin E had an effect on the expression of the *DNMTs* by semi-quantitative RT-PCR. No significant effects on the transcript levels of the three DNMTs were observed in cells treated with 100 mM vitamin E compared with untreated cells (supplementary figure 5).

Discussion

MAPT polymorphisms have been consistently linked to increased risk of PD in multiple studies.^{3-5,7,25,26} DNA methylation, with its potential to modulate gene expression, is an unexplored mechanism for the pathogenic effect of *MAPT* in PD aetiology. We have found significant evidence that abnormal methylation of *MAPT* is involved in age of onset of disease, and disease status in idiopathic PD in our cohort of 1442 leukocyte and 109 brain

tissue. This abnormal methylation of *MAPT* may not be due to alteration of genome-wide methylation, as suggested by the lack of correlation of *MAPT* methylation with global methylation levels (supplementary figure 3).

Analysis of leukocytes from neurologically normal subjects demonstrated a significant correlation of *MAPT* promoter methylation with gender and *MAPT* diplotype, (figure 1B; supplementary table 1 and 2). As the Leukocyte cohort had power of > 0.99 to detect an *a priori* small effect size of 0.1 (supplementary materials and methods), we have interpreted our negative findings for the effects of age and disease status on *MAPT* methylation as robust. Our data indicate that females have significantly higher levels of *MAPT* methylation in the leukocyte cohort (figure 1B and C). This is consistent with another study, which examined the effects of gender-specific genes that displayed opposite effects of methylation.²⁷ Women have a lower risk of developing PD than men^{28, 29} and the gender-specific methylation pattern at the *MAPT* locus observed in this study could provide an explanation for why this is the case.

Multiple studies have also reported allelic skewing in DNA methylation^{30, 31}. Our *ex-vivo* haplotype-specific pyrosequencing analysis confirmed that the H1 haplotype showed significantly higher methylation in the SK-N-F1 cell line, normal leukocyte, cerebellum and putamen DNAs compared with the H2 haplotype (figure 2). It is unclear why the H1 haplotype is more methylated than the H2. The current hypothesis of allelic skewing in methylation is that differences in CpG dinucleotide numbers lead to a cascade effect on methylation in neighbouring CpG regions, so that haplotypes with higher numbers of CpGs show higher overall methylation.³² Examination of the *MAPT* promoter region (UCSC chr17: 43,967,561 – 43,974,950) revealed that the *MAPT* CpG island has four polymorphic CpGs, two of which are associated with the H1 haplotype (rs74496580 and rs116846742) and two are associated with the H2 haplotype (rs62056781 and rs62056784) (figure 1A). The closest CpG polymorphism (rs62056776 - 1462bp distal to the CpG island) to our pyrosequenced region is associated with the H1 haplotype (figure 1A), although the functional consequence of this polymorphism remains to be determined.

To determine the effect of methylation on *MAPT* expression we examined *MAPT* DNA methylation and expression in normal cerebellum (figure 3A). Here, we observed a significant correlation between higher levels of DNA methylation and lower levels of *MAPT* expression. This pattern was also observed in our luciferase reporter assay, with methylated *MAPT* promoter constructs driving 1.3 to 1.4 fold lower expression than their unmethylated counterparts (figure 3B). This supports the paradigm that promoter hypermethylation is capable of silencing downstream gene expression.³³

We observed a significant positive correlation between methylation level and age at onset in PD cases (figure 3C). Our regression curve predicted that a higher level of methylation was associated with a later age of PD onset, where an increase in absolute methylation of 2.9 percentage points was associated with a delay of onset of 1 year. This suggests that potential therapies with modest effects on methylation levels could have a major impact on delaying the onset of disease.

Whilst levodopa had a significant effect on *MAPT* methylation in leukocytes (supplementary figure 3), it is unlikely that drug treatment was a confounding factor in our analyses. Firstly, the correlation between age-at-onset and *MAPT* methylation was robust to the inclusion of drug dosage as a covariate (multiple linear regression, $b = 0.17$, $p = 0.005$). Secondly, the effect size was relatively small, with the regression curve predicting that an increase in dosage of 1000 mg/day would decrease *MAPT* methylation by 1.4 percentage points, compared to the maximal differences in methylation levels of ~ 10 percentage points observed in our cohorts (figure 1 and 3). Finally, in our analysis of brain-region specific effects (figure 3D), we normalized the data against the mean brain *MAPT* methylation value for each case. This analysis, being a within-subjects comparison, is therefore independent of levodopa dosage and other demographic factors.

Much interest surrounds the role of micronutrients in modifying the risk for neurodegeneration, and as a therapeutic strategy for neurodegenerative disorders.³⁴ Epidemiological studies show an association between micronutrient intake and PD risk, with a recent meta-analysis of six observational studies revealing a protective effect of moderate and high vitamin E intake.³⁵ Our data on vitamin E (figure 4) is an example of a gene (H1/H2 haplotype) x environment (vitamin E levels) interaction, postulated to be a major pathogenic mechanism for PD,³⁶ and also suggests the potential for this micronutrient to alter *MAPT* methylation in carriers of the H2 haplotype *in vivo*.

Aberrant expression of a DNA methyltransferase has been linked to atypical DNA methylation levels in frontotemporal dementia, another neurodegenerative disease in which *MAPT* plays a causal role.³⁷ No significant effects were observed in cells treated with 100 μ M vitamin E on the transcript levels of the three known DNMTs (supplementary figure 5). Other mechanisms, such the effect of vitamin E on oxidative stress and downstream changes in DNA methylation,³⁸ remain to be investigated.

MAPT methylation levels were examined in three brain regions that differ in their involvement in PD pathology: the putamen, which exhibits high levels of PD pathology; the ACC, which has moderate levels of pathology; and the cerebellum, which is normally spared in PD.²¹ These regions typically do not have major neuronal cell loss,²¹ so differences in DNA methylation levels are more likely to be due to disease effects and not artifacts of neuronal loss. The brain region-specific differences in *MAPT* methylation levels in normal individuals (figure 3D), was consistent with reported regional brain differences in *MAPT* mRNA levels, with *MAPT* methylation levels (putamen > cerebellum > ACC) negatively correlated with transcript levels in the same regions (cortex > cerebellum > putamen).⁹ We hypothesise that the increase in *MAPT* methylation and associated decrease in gene expression may be a compensatory mechanism triggered during disease initiation and/or progression. This compensatory increase in methylation may be successful in regions such as the cerebellum, reducing any disease effect, but in other regions such as the putamen, there is a dysregulation of this mechanism resulting in a greater disease pathology in PD. *MAPT* methylation as a compensatory mechanism to reduce *MAPT* expression levels may also explain why PD cases with higher levels of *MAPT* methylation tend to show a later age at onset (figure 3C) and why the H1 haplotype is associated with higher levels of methylation in control cohorts (figure 1B).

At present, the relationship between *MAPT* expression levels and pathogenicity in PD is unclear. The H1 haplotype leads to increased *MAPT* expression compared with the H2 haplotype^{7,24} and has been postulated to be the underlying mechanism behind the associated increased risk of PD in H1 carriers.^{39,40} However, a transgenic mouse over-expressing a mutant *SNCA* gene showed increased, rather than decreased, pathology when crossed with a *mapt* knockout mouse.⁴¹ Moreover, it is difficult to reconcile the different methylation patterns in blood and brain DNA given they are from different subjects, a concept that requires further work. Nonetheless, our data from leukocyte and brain DNA methylation suggest that hypermethylation of the *MAPT* promoter is neuroprotective by reducing *MAPT* expression, and which could be modified by vitamin E in a haplotype-specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the National Health and Medical Research Council of Australia (NHMRC) seeding fund APP1021269, Program Grant 350833 and Capacity Building Grant 568940. GH has a NHMRC Senior Principal Research Fellowship 630434. Brain tissue was received from the Australian Brain Bank Network from brain donor programs in New South Wales (Sydney Brain Bank and New South Wales Tissue Resource Centre), Queensland, South Australia, Victoria and Western Australia. The Network is supported by the NHMRC, Neurosciences Australia, Neuroscience Research Australia, Universities of New South Wales, Sydney and Melbourne, Schizophrenia Research Institute, NIH (NIAAA) R24AA012725, Flinders Medical Centre Foundation, Mental Health Research Institute of Victoria, the Alfred Hospital, and the Victorian Forensic Institute of Medicine. We would also like to acknowledge Rebecca L. Cooper Research Laboratories at the Mental Health Research Institute of Victoria and the Bosch Molecular Biology Research Facility, University of Sydney, Sydney, NSW, Australia. Sydney MAS DNA was extracted by Genetic Repositories Australia, which is funded by NHMRC Enabling Grant 401184. We thank Prof Rehli for his kind gift of the pCpGL vector. We would also like to thank the participants of the Sydney Memory and Ageing Study and the donors of the Brain Bank. We would also like to express our thanks to the staff of the various studies.

References

1. Bertram L, Tanzi RE. The genetic epidemiology of neurodegenerative disease. *Journal of Clinical Investigation*. 2005; 115:1449–1457. [PubMed: 15931380]
2. Dickson DW, Braak H, Duda JE, et al. Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. *Lancet Neurology*. 2009; 8:1150–1157.
3. Houlden H, Singleton AB. The genetics and neuropathology of Parkinson's disease. *Acta Neuropathol*. 2012; 124:325–338. [PubMed: 22806825]
4. Simon-Sanchez J, Schulte C, Bras JM, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nature Genetics*. 2009; 41:1308–1312. [PubMed: 19915575]
5. Edwards TL, Scott WK, Almonte C, et al. Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. *Ann Hum Genet*. 2010; 74:97–109. [PubMed: 20070850]
6. Pittman AM, Myers AJ, Abou-Sleiman P, et al. Linkage disequilibrium fine mapping and haplotype association analysis of the tau gene in progressive supranuclear palsy and corticobasal degeneration. *J Med Genet*. 2005; 42:837–846. [PubMed: 15792962]
7. Kwok JBJ, Teber ET, Loy C, et al. Tau haplotypes regulate transcription and are associated with Parkinson's disease. *Ann Neurol*. 2004; 55:329–334. [PubMed: 14991810]
8. Skipper L, Wilkes K, Toft M, et al. Linkage disequilibrium and association of MAPT H1 in Parkinson disease. *Am J Hum Genet*. 2004; 75:669–677. [PubMed: 15297935]

9. Trabzuni D, Wray S, Vandrovcova J, et al. MAPT expression and splicing is differentially regulated by brain region: relation to genotype and implication for tauopathies. *Human Molecular Genetics*. 2012; 21:4094–4103. [PubMed: 22723018]
10. Caffrey TM, Wade-Martins R. Functional MAPT haplotypes: bridging the gap between genotype and neuropathology. *Neurobiology of Disease*. 2007; 27:1–10. [PubMed: 17555970]
11. Handel AE, Ebers GC, Ramagopalan SV. Epigenetics: molecular mechanisms and implications for disease. *Trends in Molecular Medicine*. 2010; 16:7–16. [PubMed: 20022812]
12. Schofield PN, Joyce JA, Lam WK, et al. Genomic imprinting and cancer; new paradigms in the genetics of neoplasia. *Toxicol Lett*. 2001; 120:151–160. [PubMed: 11323172]
13. Kwok JBJ. Role of epigenetics in Alzheimer's and Parkinson's disease. *Epigenomics-Uk*. 2010; 2:671–682.
14. Mastroeni D, McKee A, Grover A, et al. Epigenetic Differences in Cortical Neurons from a Pair of Monozygotic Twins Discordant for Alzheimer's Disease. *Plos One*. 2009; 4:e6617. [PubMed: 19672297]
15. Jowaed A, Schmitt I, Kaut O, Wullner U. Methylation Regulates Alpha-Synuclein Expression and Is Decreased in Parkinson's Disease Patients' Brains. *Journal of Neuroscience*. 2010; 30:6355–6359. [PubMed: 20445061]
16. Matsumoto L, Takuma H, Tamaoka A, et al. CpG Demethylation Enhances Alpha-Synuclein Expression and Affects the Pathogenesis of Parkinson's Disease. *Plos One*. 2010; 5:e15522. [PubMed: 21124796]
17. Tohgi H, Utsugisawa K, Nagane Y, et al. The methylation status of cytosines in a tau gene promoter region alters with age to downregulate transcriptional activity in human cerebral cortex. *Neurosci Lett*. 1999; 275:89–92. [PubMed: 10568506]
18. Barrachina M, Ferrer I. DNA methylation of Alzheimer disease and tauopathy-related genes in postmortem brain. *Journal of Neuropathology and Experimental Neurology*. 2009; 68:880–891. [PubMed: 19606065]
19. Sachdev PS, Brodaty H, Reppermund S, et al. The Sydney Memory and Ageing Study (MAS): methodology and baseline medical and neuropsychiatric characteristics of an elderly epidemiological non-demented cohort of Australians aged 70-90 years. *Int Psychogeriatr*. 2010; 22:1248–1264. [PubMed: 20637138]
20. Kwok JB, Hallupp M, Loy CT, et al. GSK3B polymorphisms alter transcription and splicing in Parkinson's disease. *Ann Neurol*. 2005; 58:829–839. [PubMed: 16315267]
21. Braak H, Ghebremedhin E, Rub U, et al. Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res*. 2004; 318:121–134. [PubMed: 15338272]
22. Wong HL, Byun HM, Kwan JM, et al. Rapid and quantitative method of allele-specific DNA methylation analysis. *Biotechniques*. 2006; 41:734–739. [PubMed: 17191619]
23. Klug M, Rehli M. Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. *Epigenetics*. 2006; 1:127–130. [PubMed: 17965610]
24. Müller T, Woitalla D, Hauptmann B, et al. Decrease of methionine and S-adenosylmethionine and increase of homocysteine in treated patients with Parkinson's disease. *Neurosci Lett*. 2001; 308:54–56. [PubMed: 11445284]
25. Kwok JBJ, Loy CT, Hamilton G, et al. Glycogen Synthase Kinase-3 beta and Tau Genes Interact in Alzheimer's Disease. *Ann Neurol*. 2008; 64:446–454. [PubMed: 18991351]
26. Pastor P, Ezquerro M, Munoz E, et al. Significant association between the tau gene A0/A0 genotype and Parkinson's disease. *Ann Neurol*. 2000; 47:242–245. [PubMed: 10665497]
27. Pankratz N, Wilk JB, Latourelle JC, et al. Genomewide association study for susceptibility genes contributing to familial Parkinson disease. *Human Genetics*. 2009; 124:593–605. [PubMed: 18985386]
28. Boks MP, Derks EM, Weisenberger DJ, et al. The Relationship of DNA Methylation with Age, Gender and Genotype in Twins and Healthy Controls. *Plos One*. 2009; 4
29. Haaxma CA, Borm GF, Bloem BR, Horstink MWIM. Gender differences in Parkinson's disease. *Movement Disord*. 2006; 21:S84–S84.
30. Schilling E, El Chartouni C, Rehli M. Allele-specific DNA methylation in mouse strains is mainly determined by cis-acting sequences. *Genome Res*. 2009; 19:2028–2035. [PubMed: 19687144]

31. Schalkwyk LC, Meaburn EL, Smith R, et al. Allelic Skewing of DNA Methylation Is Widespread across the Genome. *Am J Hum Genet.* 2010; 86:196–212. [PubMed: 20159110]
32. Kerkel K, Spadola A, Yuan E, et al. Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. *Nature Genetics.* 2008; 40:904–908. [PubMed: 18568024]
33. Kass SU, Landsberger N, Wolffe AP. DNA methylation directs a time dependent repression of transcription initiation. *Curr Biol.* 1997; 7:157–165. [PubMed: 9395433]
34. Gillette-Guyonnet S, Secher M, Vellas B. Nutrition and neurodegeneration: epidemiological evidence and challenges for future research. *Br J Clin Pharmacol.* 2013; 75:738–755. [PubMed: 23384081]
35. Etminan M, Gill SS, Samii A. Intake of vitamin E, vitamin C, and carotenoids and the risk of Parkinson's disease: a meta-analysis. *Lancet Neurol.* 2005; 4:362–365. [PubMed: 15907740]
36. Cannon JR, Greenamyre JT. Gene-environment interactions in Parkinson's disease: Specific evidence in humans and mammalian models. *Neurobiol Dis.* 2012 Epub ahead of print.
37. Banzhaf-Strathmann J, Claus R, Mucke O, et al. Promoter DNA methylation regulates progranulin expression and is altered in FTLD. *Acta Neuropathologica Comm.* 2013; 1:16.
38. Switzeny OJ, Müllner E, Wagner KH, et al. Vitamin and antioxidant rich diet increases MLH1 promoter DNA methylation in DMT2 subjects. *Clin Epigenetics.* 2012; 4:19. [PubMed: 23025454]
39. Tobin JE, Latourelle JC, Lew MF, et al. Haplotypes and gene expression implicate the MAPT region for Parkinson disease: the GenePD Study. *Neurology.* 2008; 71:28–34. [PubMed: 18509094]
40. Hayesmoore JBG, Bray NJ, Cross WC, et al. The effect of age and the H1c MAPT haplotype on MAPT expression. *Neurobiology of Aging.* 2009; 30:1652–1656. [PubMed: 18276036]
41. Morris M, Koyama A, Masliah E, Mucke L. Tau reduction does not prevent motor deficits in two mouse models of Parkinson's disease. *Plos One.* 2011; 6:e29257. [PubMed: 22206005]

Abbreviations

DNMT	DNA methyltransferase
MAPT	microtubule-associated protein tau
PD	Parkinson's disease
ACC	anterior cingulate cortex
SNP	single nucleotide polymorphisms

Figure 1.

Schematic diagram of the *MAPT* promoter region and CpG island. (A). Polymorphisms predicted to form CpG dinucleotides corresponding to either the H1 or H2 haplotype. The region examined by the pyrosequencing assay (inverted triangle), and the polymorphism used to differentiate between H1 and H2 haplotype are indicated. Effect of *MAPT* diplotype and gender on *MAPT* promoter methylation in leukocyte DNA from (B) controls, (C) PD individuals. *** = $p < 0.0001$.

Figure 2.

Haplotype-specific pyrosequencing of *MAPT* methylation. Methylation levels for the two haplotypes in each sample are indicated as paired data. Tissues examined were leukocyte, cerebellum, anterior cingulate cortex (ACC) and putamen. * = $p < 0.05$.

Figure 3.

Effect of *MAPT* methylation on gene expression. (A) Scatter plot of *MAPT* methylation versus *MAPT* expression levels in cerebellum samples of Brain1 cohort (N = 69). Effect of *MAPT* methylation on PD. (B) In vitro analysis of relative expression in unmethylated (white) versus methylated (black) luciferase reporter constructs in SK-N-F1 cell. Error bars indicate standard error of the mean from five independent experiments. (C) Scatter plot of methylation versus PD age of onset from Leukocyte cohort (N=358). (D) Analysis of relative *MAPT* methylation in control (white, N = 12) and PD (black, N = 28) patients in cerebellum (W = 185, $p = 0.034$), anterior cingulate cortex (W = 210.5, $p = 0.151$) and putamen (W = 384.5, $p = 0.001$) from Brain2 cohort. Error bars indicate standard error of the mean. * = $p < 0.05$, ** = $p < 0.001$.

Figure 4.

Effect of vitamin E on *MAPT* methylation. A + B) Scatter plots of vitamin E versus *MAPT* methylation levels in individuals segregated by their diplotype status as H1 (H1/H1 homozygotes) and H2 carriers (H1/H2 heterozygotes and H2/H2 heterozygotes). C) Dose response effect of vitamin E on either the H1 (open bar) or H2 (grey bar) of *MAPT* in SK-N-F1 cells. Methylation levels of each replicate were normalized against the value observed for the H1 haplotype at 0 mM vitamin E. Error bars indicate standard error of the mean from five independent experiments * = $p < 0.05$, ** = $p < 0.001$.

Table 1

Comparisons of demographics and *MAPT* diplotype frequencies in cohorts studied

Cohort	Tissue	Disease status	Sex			Age ^a	Age Range	MAPT Diplotype					MAPT Methylation Levels ^a	p value ^b	H2/H2	H1/H2	H1/H1	p value ^c	p value ^c
			N	M	F														
Leukocyte	Leukocyte	Normal	1084	480 (44%)	604 (56%)	76.9 ± 7	42-99	623 (58%)	384 (35%)	71 (7%)	> 0.05 [*]	23.7 ± 6	> 0.05	23.0 ± 6	17 (4%)	113 (32%)	228 (64%)	< 0.001	42-99
		PD	358	219 (61%)	139 (39%)	72.0 ± 9	45-106	228 (64%)	113 (32%)	17 (4%)		23.0 ± 6							
Brain1	brain region	Normal	69	52 (75%)	17 (25%)	46.2 ± 17	21-80	41 (59%)	26 (38%)	2 (3%)		5.8 ± 2		17.0 ± 5	2 (7%)	4 (14%)	22 (79%)	> 0.05	65-93
		Normal	12	8 (67%)	4 (33%)	78.8 ± 9	65-93	12 (58%)	5 (42%)	0 (0%)	> 0.05	17.0 ± 5	> 0.05						
Brain2	brain regions	Normal	28	16 (57%)	12 (42%)	80.7 ± 6	69-95	22 (79%)	4 (14%)	2 (7%)		16.0 ± 6		16.0 ± 6	2 (7%)	4 (14%)	22 (79%)	> 0.05	69-95
		PD																	

^aMean ± SD

^bPearson chi square test for comparisons of gender and diplotype frequencies between cases and controls.

^cStudent's *t* test for mean differences in age (at collection) and *MAPT* methylation between cases and controls.

^{*}Logistic regression analysis with age and gender as co-variables gave a p value of 0.043.