Tau levels do not influence human ALS or motor neuron degeneration in the *SOD1*^{G93A} mouse

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ABSTRACT

Background: The microtubule-associated protein tau is thought to play a pivotal role in neurodegeneration. Mutations in the tau coding gene *MAPT* are a cause of frontotemporal dementia, and the H1/H1 genotype of *MAPT*, giving rise to higher tau expression levels, is associated with progressive supranuclear palsy, corticobasal degeneration, and Parkinson disease (PD). Furthermore, tau hyperphosphorylation and aggregation is a hallmark of Alzheimer disease (AD), and reducing endogenous tau has been reported to ameliorate cognitive impairment in a mouse model for AD. Tau hyperphosphorylation and aggregation have also been described in amyotrophic lateral sclerosis (ALS), both in human patients and in the mutant *SOD1* mouse model for this disease. However, the precise role of tau in motor neuron degeneration remains uncertain.

Methods: The possible association between ALS and the *MAPT* H1/H2 polymorphism was studied in 3,540 patients with ALS and 8,753 controls. Furthermore, the role of tau in the *SOD1*^{G93A} mouse model for ALS was studied by deleting *Mapt* in this model.

Results: The *MAPT* genotype of the H1/H2 polymorphism did not influence ALS susceptibility (odds ratio = 1.08 [95% confidence interval 0.99–1.18], *p* = 0.08) and did not affect the clinical phenotype. Lowering tau levels in the *SOD1*^{G93A} mouse failed to delay disease onset (*p* = 0.302) or to increase survival (*p* = 0.557).

Conclusion: These findings suggest that the H1/H2 polymorphism in *MAPT* is not associated with human amyotrophic lateral sclerosis, and that lowering tau levels in the mutant *SOD1* mouse does not affect the motor neuron degeneration in these animals. *Neurology*® 2010;74:1687–1693

GLOSSARY

AD = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **ANOVA** = analysis of variance; **CI** = confidence interval; **hAPP** = human amyloid precursor protein; **CBD** = corticobasal degeneration; **eGFP** = enhanced green fluorescent protein; **FTD** = frontotemporal dementia; **MAPT** = microtubule-associated protein tau; **OR** = odds ratio; **PD** = Parkinson disease; **PFA** = paraformaldehyde; **PSP** = progressive supranuclear palsy; **SNP** = single nucleotide polymorphism; **SOD1** = superoxide dismutase 1.

Mutations in proteins involved in axonal structure and function are a well-known cause of neurodegenerative disorders. Mutations in the gene coding for the microtubule-associated protein tau (*MAPT*) are a cause of familial frontotemporal dementia (FTD) with or without amyotrophy.^{1–3} Furthermore, an association has been found between the *MAPT* H1/H1 genotype and an increased occurrence of progressive supranuclear palsy (PSP),^{4–6} corticobasal degeneration (CBD),^{5,7} and Parkinson disease (PD).^{8,9} Finally, hyperphosphorylated tau in the neurofibrillary tangles is a hallmark of Alzheimer disease (AD). Interestingly, deletion of *Mapt* from a mouse model for AD attenuates the pathology in these animals, suggesting that tau levels play a role in the mechanism of β -amyloid-induced neurodegeneration.¹⁰ Similarly, a possible mechanism that mediates the genetic association between the H1/H2 polymorphism and neurodegenerative disease may be the fact that alleles that occur on the H1 haplotype are associated with higher tau transcript expression,¹¹ although other effects have been reported.^{6,12}

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We studied the significance of tau expression levels for the pathogenesis of motor neuron degeneration in amyotrophic lateral sclerosis (ALS) by studying the possible association of the H1/H2 polymorphism and ALS in different large and well-defined sporadic ALS patient populations, and by investigating the effect of the deletion of *Mapt* from the transgenic mouse model for ALS, the *SOD1*^{G93A} mouse.

METHODS Subjects. A total of 3,540 patients with ALS from 7 study populations were diagnosed according to the El Escorial criteria after full investigation; cases with a family history of ALS were excluded. Control individuals were recruited from the same populations as the patients. Part of the US population has been described previously.¹³ The Dutch population overlaps with that described previously in van Es et al.¹⁴ UK controls additionally included data on 2,938 individuals typed as part of the Wellcome Trust Case Control Consortium. Demographic information of patients and controls is supplied in appendix e-1 on the *Neurology*[®] Web site at www.neurology.org.

Genotyping. The H1/H2 polymorphism was tagged by SNP rs9468¹⁵ (Belgium and Poland), and genotyped with a Taqman Assay-on-Demand (C_7563752_10) on a 7300 Sequence Detection System (Applied Biosystems), or by SNP rs8070723 and genotyped as part of the Illumina 317K, 370K, or 550K (the Netherlands, Sweden, United Kingdom, United States, Italy) or Affymetrix 500K (UK WTCCC controls) panels. The 2 SNPs have an r^2 of 1 within the HapMap CEU population.

Mouse breeding. Mice with a homozygous or heterozygous deletion of tau [*Mapt*^{tm1(EGFP)Kl/J}] were purchased from The Jackson Laboratory (Bar Harbor, ME). The endogenous tau is inactivated by an insertion of an enhanced green fluorescent protein (eGFP) coding sequence in the first exon of the *Mapt* gene.¹⁶ These mice were backcrossed for at least 5 generations with non-transgenic C57Bl6 mice to further increase their C57Bl6 background. Female *Mapt*^{+/-} mice were crossed with male mice overexpressing human mutant *SOD1* [B6SJL-TgN(SOD1-G93A)1Gur; The Jackson Laboratory] that were crossed into a C57Bl6 background for more than 20 generations. Male *SOD1*^{G93A}/*Mapt*^{+/-} mice were then crossed with female *Mapt*^{+/-} mice to obtain the following genotypes: *SOD1*^{G93A}/*Mapt*^{+/+}, *SOD1*^{G93A}/*Mapt*^{+/-}, *SOD1*^{G93A}/*Mapt*^{-/-}. All mice were genotyped by PCR on DNA from tail biopsies by using the primers IMR0872, IMR873, IMR3092, and IMR3093 for *Mapt* genotyping and the primers IMR042, IMR043, IMR113, and IMR114 for the genotyping of *SOD1*^{G93A} (Invitrogen, Carlsbad, CA; table e-1).

Determination of disease onset and survival. Disease onset was determined as previously described.¹⁷ In short, mice were trained to walk on a rotarod (Ugo Basile, model 7600) at 15 rpm for at least 3 minutes. Motor performance was evaluated twice a week. An investigator blinded to the genotypes of the mice monitored the time of latency to fall, of which the maximum was set at 3 minutes. Each trial consisted of 5 successive rounds of which an average was made. When the average dropped permanently below 60 seconds, the threshold for disease onset was reached. Survival was determined as before by a

blinded investigator,¹⁷ by laying down the mouse on its back and monitoring the time the mouse needed to roll over. If this took longer than 30 seconds, the mouse was killed and this time point was considered as the time of death. Both disease onset and survival are presented as Kaplan-Meier curves.

Immunoblotting. Spinal cords and brains of symptomatic mice (115 days old) were homogenized with Lysing Matrix A (MP Biomedicals, Irvine, CA) and protein concentration was determined using a Micro BCA protein kit (Pierce, Rockford, IL). Of each sample the same amount of protein was loaded on a 4%–12% BisTris gel (Invitrogen) and then blotted on a Immobilon-P transfer membrane (Millipore, Bedford, MA). The primary antibodies used were anti-tau1 antibody (Millipore, Billerica, MA), anti-eGFP antibody (Invitrogen), anti-human SOD1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti- β -actin (Sigma, St. Louis, MO). The secondary antibodies were anti-goat for detection of human SOD1, anti-rabbit for detection of eGFP, and anti-mouse for detection of tau and β -actin (Sigma, St. Louis, MO). The immunostaining of β -actin served as loading control. The fluorescence signal was generated with ECF substrate (GE Healthcare, Munich, Germany) and detected with a STORM 840 scanner (Molecular Dynamics, Sunnyvale, CA). The intensity of bands was analyzed with ImageQuant software (Molecular Dynamics).

Quantification of motor neuron loss. Histologic analysis of spinal cords was performed as previously described.¹⁷ In brief, symptomatic mice (120 days old) were killed using CO₂ immediately followed by transcardiac perfusion with PBS and PBS with 4% paraformaldehyde (PFA). The lumbar region of the spinal cord was removed, further fixed with 4% PFA, and embedded in paraffin. Every tenth section of 7- μ m thickness was deparaffinated and stained with hematoxylin and eosin. At 20 $\times$ magnification, the area of normal-appearing neurons with nucleoli in the ventral horn was calculated using Nis-Elements (version AR 2.30, Nikon Instruments Inc., NY) and the number of neurons in different size groups was determined.

Standard protocol approvals, registrations, and patient consents. All patients gave informed consent as approved by the local ethical committees. All animal experiments were approved by the local ethical committee of the KULeuven (n°P07040).

Statistical analysis. Association analysis was performed with the PLINK package. Cochran-Mantel-Haenszel test for genotype was performed and graphically plotted with StatsDirect. Survival analysis was done with the Cox regression method, taking into account gender, age at onset, site of onset, and country as covariates, and comparison of age at onset with linear regression taking into account gender, site of onset, and country as covariates, in SPSS 13.0.

The combined study population has >80% power to detect with nominal significance ($p = 0.05$) an odds ratio (OR) of H1/H1 compared to H2 carriers of ≥ 1.12 and 95% power to exclude an effect with $OR \geq 1.16$.

Analyses of mouse studies were performed using StatsDirect and SPSS 13.0 software. Survival was analyzed by log-rank, disease onset by analysis of variance (ANOVA). Pathologic data were analyzed by ANOVA for each size group. α was a priori set at 0.05. This study has 80% power to detect a difference in survival of ≥ 11 days and 90% power for a difference of ≥ 13 days at the 5% significance level.

Table 1 Association of amyotrophic lateral sclerosis with H1/H1 vs H2 carriage

Stratum	Cases	Controls	SNP ^a	Missingness (%) ^b	HWE case/ <i>p</i> ^c	HWE ctrl/ <i>p</i> ^d	H1/H1 freq cases (counts)	H1/H1 freq controls (counts)	OR	95% CI	<i>p</i>
BEL	343	1,364	rs9468	1.23	0.76	0.26	60.5 (207/342)	57.1 (767/1,344)	1.15	0.9-1.48	0.25
NL	1,058	1,066	rs8070723	0.00	0.13	0.07	59.8 (633/1,058)	58.2 (620/1,066)	1.07	0.90-1.28	0.43
ITA	266	1,189	rs8070723	0.00	0.66	0.56	50.8 (135/266)	52.3 (622/1,189)	0.94	0.71-1.24	0.65
POL	210	561	rs9468	4.41	0.38	0.38	73.5 (147/200)	68.2 (366/537)	1.30	0.89-1.90	0.16
SWE	476	486	rs8070723	0.00	1.00	1.00	72.7 (346/476)	73.3 (356/486)	0.97	0.72-1.31	0.84
US	949	937	rs8070723	0.00	0.69	0.51	62.4 (592/949)	59.6 (558/937)	1.13	0.93-1.36	0.21
UK	238	3,150	rs8070723	0.59	1.00	0.69	60.3 (143/237)	58.3 (1,824/3,131)	1.09	0.83-1.44	0.53
Overall	3,540	8,753	—	0.58	0.54	0.90	—	—	1.08	0.99-1.18	0.08

Abbreviations: BEL = Belgium; CI = confidence interval; HWE = Hardy-Weinberg equilibrium; ITA = Italy; NL = the Netherlands; OR = odds ratio; POL = Poland; SNP = single nucleotide polymorphism; SWE = Sweden; UK = United Kingdom; US = United States.

^a H1/H2 tagging SNP.

^b Genotype missing rate, *p* value for deviation from Hardy-Weinberg equilibrium in cases^c and controls^d.

RESULTS Association study of MAPT polymorphism with human ALS.

A total of 3,540 patients with ALS and 8,753 controls originating from 7 different cohorts were genotyped for the *MAPT* inversion polymorphism (H1/H2). Marker statistics and association measures for each study population are shown in table 1. No heterogeneity in the OR was observed between studies (Breslow-Day statistic $p = 0.80$). In none of the populations was a significant increase in the frequency of the H1/H1 genotype in patients with ALS compared to controls found, as shown in table 1 and figure 1. A combined analysis showed no association [OR = 1.08 (95% confidence interval [CI] 0.99–1.18), $p = 0.08$]. Thus, in spite of having a power of more than 80% to detect an OR of 1.12 at the 5% significance level, no association was found.

Figure 1 Meta-analysis of association of amyotrophic lateral sclerosis with H1/H1 vs H2 carriage in all available populations

Numbers on the right represent the odds ratio and the 95% confidence interval per population. BEL = Belgium; CI = confidence interval; ITA = Italy; NL = the Netherlands; POL = Poland; SWE = Sweden; UK = United Kingdom; US = United States.

We then examined whether the *MAPT* genotype was associated with disease parameters such as age at onset and survival. Age at onset data (table 2) were available for 1,540 patients and survival data for 1,307 patients. Whereas age at onset ($p = 2 \times 10^{-22}$) and site of onset ($p = 2 \times 10^{-6}$) were associated with survival as expected, there was no such association with *MAPT* genotype ($p = 0.832$) (figure 2).

Effect of reduction of tau levels on motor neuron degeneration in the *SOD1*^{G93A} mouse.

We next investigated the effect of reduction of tau expression on the motor neuron degeneration in the *SOD1*^{G93A} mouse, a thoroughly studied rodent model for human ALS. *SOD1*^{G93A} mice were crossbred with tau knock-out mice (*Mapt*^{-/-}) to generate *SOD1*^{G93A}/*Mapt*^{+/-}, *SOD1*^{G93A}/*Mapt*^{+/-}, and *SOD1*^{G93A}/*Mapt*^{-/-} mice. Immunoblotting confirmed that tau protein was absent in the spinal cord and brain of *Mapt*^{-/-} mice and its levels reduced in the spinal cord and brain of *Mapt*^{+/-} mice compared to *Mapt*^{+/-} mice (figure 3A, upper panel). Deleting *Mapt* did not affect the expression of human *SOD1*^{G93A} protein in the spinal cord of the double transgenic mice (figure 3A, lower panel). We monitored motor neuron degeneration by determining disease onset (failure of motor performance on rotarod) and survival. No difference in disease onset was observed between *SOD1*^{G93A}/*Mapt*^{+/-} mice and *SOD1*^{G93A} mice that were either *Mapt*^{+/-} or *Mapt*^{-/-} ($p = 0.302$) (figure 3B). On average, the *SOD1*^{G93A}/*Mapt*^{+/-} mice failed to walk at least 60 seconds at age 125 ± 3 days ($n = 11$), the *SOD1*^{G93A}/*Mapt*^{+/-} mice at age 130 ± 3 days ($n = 14$), and the *SOD1*^{G93A}/*Mapt*^{-/-} mice at age 122 ± 5 days ($n = 13$) (average $\pm$ SEM). The average survival was 141 ± 3 days ($n = 18$) for *SOD1*^{G93A}/*Mapt*^{+/-}

Table 2 Association of H1/H2 polymorphism with age at onset^a

	BEL	NL	SWE	US	Combined
H1/H1	58.7 ± 12.2	60.9 ± 11.3	61.2 ± 12.9	54.8 ± 12.0	59.5 ± 12.4
H1/H2	58.7 ± 13.2	61.1 ± 11.0	63.4 ± 12.1	54.7 ± 11.4	59.9 ± 12.2
H2/H2	55.9 ± 12.3	59.3 ± 8.3	59.8 ± 13.2	55.7 ± 16.4	57.8 ± 11.9

Abbreviations: BEL = Belgium; NL = the Netherlands; SWE = Sweden; UK = United Kingdom; US = United States.

^a Age at onset in years.

Mapt^{+/+}, 149 days ± 2 days (n = 21) for *SOD1*^{G93A}/*Mapt*^{+/+}, and 146 days ± 3 days (n = 19) for *SOD1*^{G93A}/*Mapt*^{-/-} mice. This difference was far from reaching significance (*p* = 0.557), and lacked dose-dependency (figure 3C). To confirm this lack of effect of reduction of tau levels on motor neuron degeneration, we counted the number of remaining motor neurons at 120 days of age in all 3 mouse groups and determined the size of their perikaryon. As depicted in figure 3, D and E, no significant difference was observed in neurodegeneration between mice with normal tau levels and mice with reduced tau levels.

DISCUSSION The tau H1/H1 genotype has previously been confirmed as associated with PSP and CBD (OR approximately 4)⁵⁻⁷ and PD (OR approximately 1.3).^{8,9} In other neurologic diseases such as AD¹⁸⁻²¹ and FTD²²⁻²⁵ results have remained inconclusive.

Hitherto, it was uncertain whether a similar association existed for ALS. Two studies have previ-

ously addressed a possible role of the H1/H2 polymorphism in ALS in populations from Germany and Guam, but both were limited by the small sample size.²⁶⁻²⁸ Moreover, Guam ALS may be pathogenetically different and the significance of Guam ALS to understand “common” ALS is uncertain.^{27,28}

Therefore, we investigated the possible association of the H1/H2 polymorphism in a large and well-defined sporadic ALS study population consisting of 3,540 cases and 8,753 controls. Although this combined study population has >80% power to detect an OR ≥ 1.12 at the 5% significance level, no association was observed. Our genetic data therefore do not suggest a significant role for *MAPT* in susceptibility to ALS, as opposed to other neurodegenerative disorders.

Previously, a dose-dependent effect of the H2 haplotype decreasing age at onset has been suggested for FTD^{29,30} and PD.³¹ We therefore investigated a possible effect of the H1/H2 polymorphism on 2 disease parameters, i.e., age at onset and disease duration. We were unable to confirm an effect of the *MAPT* genotype on age at onset. As previously described,³² age at onset and site of onset were highly correlated with survival, but *MAPT* genotype did not influence disease duration.

Our data thus do not support a contribution of a genetic polymorphism affecting tau expression levels in the pathogenesis of ALS. To confirm these findings, we studied a model in which tau expression levels could be affected experimentally to a greater extent than seen for the H1/H2 polymorphism. To this end, and because of the unexpected results reported for the AD model mentioned above,¹⁰ we investigated the effect of tau expression levels on the motor neuron degeneration in the *SOD1*^{G93A} mouse. Reduction of tau expression to 50% of normal (heterozygous mice) or complete absence of tau (homozygous mice) in *SOD1*^{G93A} mice did not influence survival (*p* = 0.557) or onset of clinical motor deficits assessed by rotarod performance (*p* = 0.302). To exclude a subtle effect on motor neuron survival that would escape behavioral or survival analyses, we quantified the number of motor neurons in the spinal cord in *SOD1*^{G93A} and double transgenic mice, but we could only confirm the lack of effect of partial or complete tau deletion. The power of this animal study was sufficient to detect a difference of ≥ 11 days by 80% and of ≥ 13 days by 90%. This result is of interest as it is in contrast with the recently reported beneficial effect of tau deletion in a mouse model for AD.¹⁰ In this study, the authors found that deletion of *Mapt* in the hAPP overex-

Figure 2 Association of H1/H2 polymorphism with survival

(A) Protein expression of human mutant *SOD1*^{G93A}, tau, and eGFP in double transgenic mice. β -actin served as loading control. (B) Probability of disease onset in *Mapt*^{+/+}, *Mapt*^{+/-}, and *Mapt*^{-/-} mutant *SOD1*^{G93A} mice ($n = 11$ – 14 per group, $p = 0.302$). (C) Probability of survival in *Mapt*^{+/+}, *Mapt*^{+/-}, and *Mapt*^{-/-} mutant *SOD1*^{G93A} mice ($n = 18$ – 21 per group, $p = 0.557$). (D) Number of neurons in the ventral horn categorized per size of *Mapt*^{+/+}, *Mapt*^{+/-}, and *Mapt*^{-/-} mutant *SOD1*^{G93A} mice ($n = 3$ per group, $p_{100-150} = 0.163$, $p_{150-200} = 0.698$, $p_{200-250} = 0.803$, $p_{>250} = 0.537$) (data are represented as average $\pm$ SEM). (E) Hematoxylin and eosin staining of the ventral horn of the lumbar spinal cord of 120-day-old *Mapt*^{+/+}, *Mapt*^{+/-}, and *Mapt*^{-/-} mutant *SOD1*^{G93A} mice. White scale bar = $100 \mu\text{m}$. White dashed line indicates the border between white matter and gray matter.

pressing mouse attenuated disease and protected neurons from excitotoxic and β -amyloid-induced cell death. Therefore, our negative result is disappointing given the known contribution of excitotoxicity to the pathogenesis of (mutant *SOD1*-associated) ALS.^{33,34} Of note, we used the same source of *Mapt*^{-/-} mice as has been used in the AD study.

Combining the data from this large-scale genetic association study and the animal study, we conclude that the H1/H2 polymorphism—affecting tau expression levels—is not associated with human ALS and that lowering tau levels in mutant *SOD1* mice

does not affect motor neuron degeneration in these mice. In this regard, ALS thus differs substantially from various other neurodegenerative diseases. In particular, our mouse study demonstrates that the effect of deletion of tau in the *hAPP* mouse is not a characteristic that can be easily extrapolated to other neurodegenerative diseases, in spite of the contribution of excitotoxicity to the motor neuron degeneration in the *SOD1*^{G93A} mice.^{33–35} Our results also suggest that strategies to reduce tau expression are unlikely to represent a general therapeutic option for neurodegenerative disorders.

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