TMCC2 Associates with Alzheimer's Disease Pathology

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ABSTRACT

We previously identified Transmembrane and Coiled-Coil 2 (TMCC2) as a protein that forms complexes with both apolipoprotein E (apoE) and the amyloid protein precursor (APP) and which displayed differential affinity for apoE isoforms apoE3 and apoE4. Here we have for the first time examined TMCC2 in the human brain and found that it is affected by APOE genotype and brain region. We further observed that TMCC2 associates with the pathology of Alzheimer's disease in dense core and neuritic plaques. TMCC2 is therefore positioned to mediate impacts of apoE4 on Alzheimer's disease pathology.

INTRODUCTION, RESULTS AND DISCUSSION

Alzheimer's Disease (AD) is the most common neurodegenerative disease and the leading cause of dementia in elderly people. Disturbed metabolism of the amyloid protein precursor (APP) or its metabolic derivatives is an established feature of AD. The ε4 isoform of apolipoprotein E (apoE4) is the greatest genetic risk factor for AD after age, having a semi-dominant pattern of inheritance, and an allele frequency of approximately 15% worldwide [1]; the impact of apoE4 on AD is thus both common and large. Compared to the most common apoE isoform, apoE3, apoE4 (Cys158 to Arg) alters the metabolism of APP, and/or its derivative the Aβ peptide, in both human studies and experimental mice [2,3]. Yet the mechanisms linking apoE, APP and/or Aβ metabolism, and risk of neurodegeneration are poorly understood.

In the presence of Transmembrane and Coiled-Coil 2 (TMCC2), apoE4 mediated an increase in Aβ secretion from cells expressing an autosomal dominant mutant form of APP, APPswedish (K595N, M596L), as well as from the C-terminal fragment of APP [4]. TMCC2 thus shows differential interactions with normal versus risk forms of two separate proteins implicated in the genetic etiology of AD.

The interaction of TMCC2 with APP is evolutionarily conserved: its Drosophila orthologue, called Dementin, is highly similar to TMCC2 in amino acid sequence and protects against ectopic expression of human APP in vivo [5]. When mutated, Dementin perturbs metabolism of the endogenous Drosophila orthologue of APP, the APP-Like protein and causes neurodegeneration with pathological features of AD, including accumulation of fragments of the APP-Like protein, synaptic defects and early death [5].

Here we report the first investigation of TMCC2 in the human brain. We observed that TMCC2 is affected by both APOE genotype and brain region and associates with the pathology of AD, implicating this protein in AD pathogenesis.

Given the differential association of TMCC2 with apoE3 and apoE4 as well as with APP, we investigated TMCC2 protein levels and cellular distribution in 10 human post-mortem AD brains homozygous for either APOE3 or APOE4 and in five APOE3 homozygous age-matched controls, (case details are provided in supplementary Table 1). Using an antibody previously validated against mouse and rat brain-derived TMCC2, (antibody 94) [4], we detected a set of three bands in human brain homogenates by western blot. The highest of these migrated at a position that would be predicted from the amino acid

composition of TMCC2 (77.5 kDa; Fig 1A). A similar pattern was observed using a separate independently generated antibody (antibody 11193), but which had reduced specificity compared to antibody 94 (Fig. S1A). The lower migrating bands thus potentially correspond to alternate or modified forms of TMCC2, hereafter referred to collectively as TMCC2*. Within either the temporal gyrus or cerebellum, total levels of TMCC2 were unaffected by APOE or AD status (Fig. S1B and S1C). However, a larger proportion of TMCC2 was found as TMCC2* in cerebellum than in temporal gyrus: of total TMCC2, 57.5 ± 2.7% of TMCC2 in the cerebellum was TMCC2*, compared to 42.5 ± 3% in the temporal gyrus ($p=0.002$ by two-tailed Student's t-test). This effect was found to be more significant in APOE3 compared to APOE4 homozygotes (Fig. 1B, full blots are shown in Figs. S1B and S1C). Levels of TMCC2* are therefore potentially driven by a higher rate of conversion of TMCC2 to TMCC2* in APOE3 homozygous brains. Thus the biology of TMCC2 in the human brain was seen to be affected by both APOE genotype and brain region.

TMCC2 immunoreactivity co-localized with APP and was detected primarily in a cellular and vesicular pattern within neurons, consistent with proposed roles of TMCC2 in endomembrane systems [4, 6] (Fig. 1C). In AD brains, we found that TMCC2 immunoreactivity is notable in the centre of dense core senile plaques detected using antibody 4G8 (Fig. 2A), but not in diffuse amyloid plaques (Fig. 2B). In contrast, immunostaining for TMCC2 and phospho-tau using antibody AT8 did not reveal an association between TMCC2 immunoreactivity and neurofibrillary tangles or neuropil threads in neuritic plaques (Fig. 2C). TMCC2 interacts with the 99-amino acid C-terminus of APP [4] that is generated in vivo following cleavage by BACE; we therefore stained AD temporal gyrus sections for TMCC2 and for APP using an antibody that binds its C-terminus (antibody C1/6.1) and for amyloid using methoxy-X04. This confirmed localization of TMCC2 immunoreactivity within dense cores as well as co-localization with APP (Figs 2D and 2E). We further observed increased expression and strong co-localization of TMCC2 and APP in dystrophic neurites surrounding neuritic plaques (Fig 2F).

Taken together, the above observations support an association between TMCC2 and APP in the human brain, complementing previous observations in vitro, in rat brain, and in Drosophila [4, 5]. Our observations further associate TMCC2 with AD pathology and APOE genotype, suggesting novel directions for research into the mechanisms by which apoE4 contributes to AD pathogenesis.

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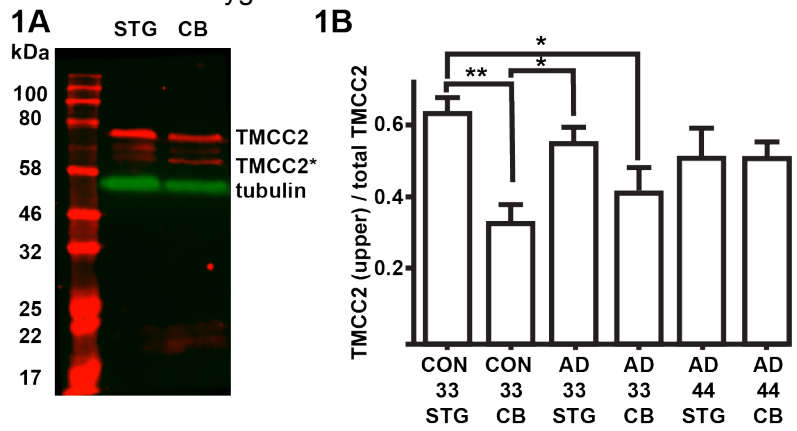
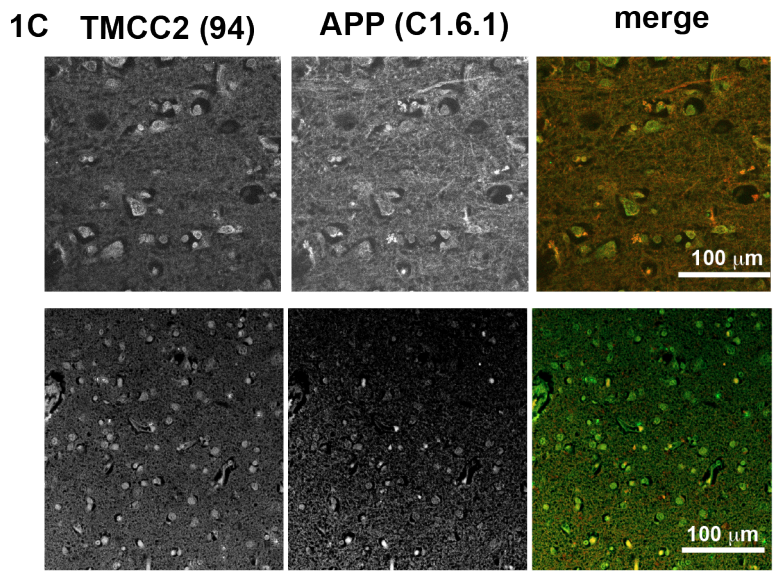
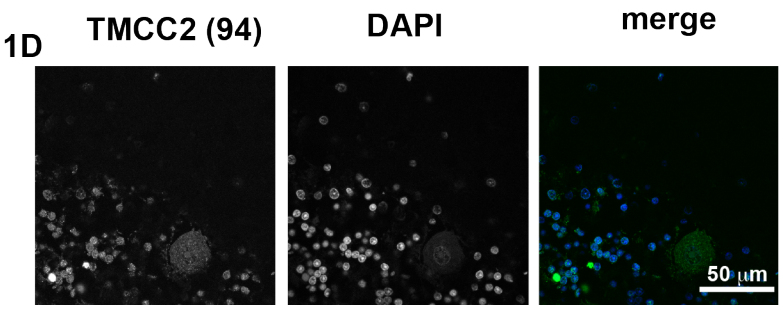
Figures and Legends

Figure 1. **Expression of TMCC2 in the human brain.** 1A, western blot of human superior temporal gyrus (STG) and cerebellum (CB) for TMCC2 detected using antibody 94; 1B, quantification of the ratio of TMCC2 (upper band) to total TMCC2 in brain specimens stratified by APOE genotype and AD status, statistical significance of differences was estimated by one-way ANOVA with Tukey's post-hoc multiple comparison test (**, p<0.005; *, p<0.05); 1C, detection of TMCC2 (antibody 94) and APP (antibody C1/6.1) in human brain superior temporal gyrus; 1D, detection of TMCC2 (antibody 94) in human cerebellum, both of APOE3 homozygous controls.







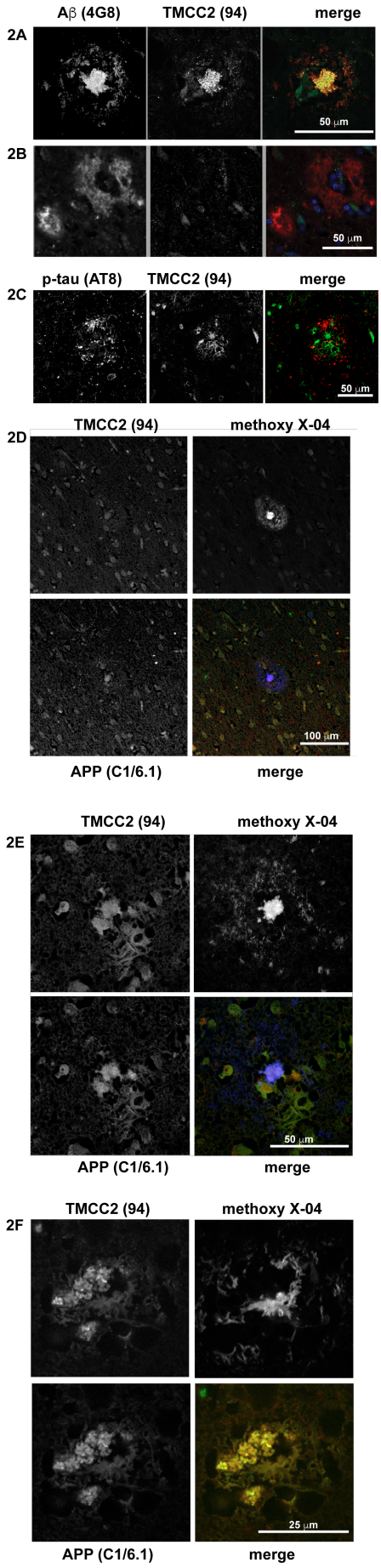


Figure 2. Association of TMCC2 with AD pathology in human superior temporal gyrus. 2A, TMCC2 immunoreactivity was detected in the centre of dense core senile plaques, but not in diffuse amyloid plaques (2B); 2C, TMCC2 did not associate with phospho-tau (AT8); 2D and 2E, TMCC2 co-localization with APP in dense core plaques detected using methoxy X04; 2F, co-localization of TMCC2 and APP in dystrophic neurites of neuritic plaques

Supplementary Materials and Methods

Human brain

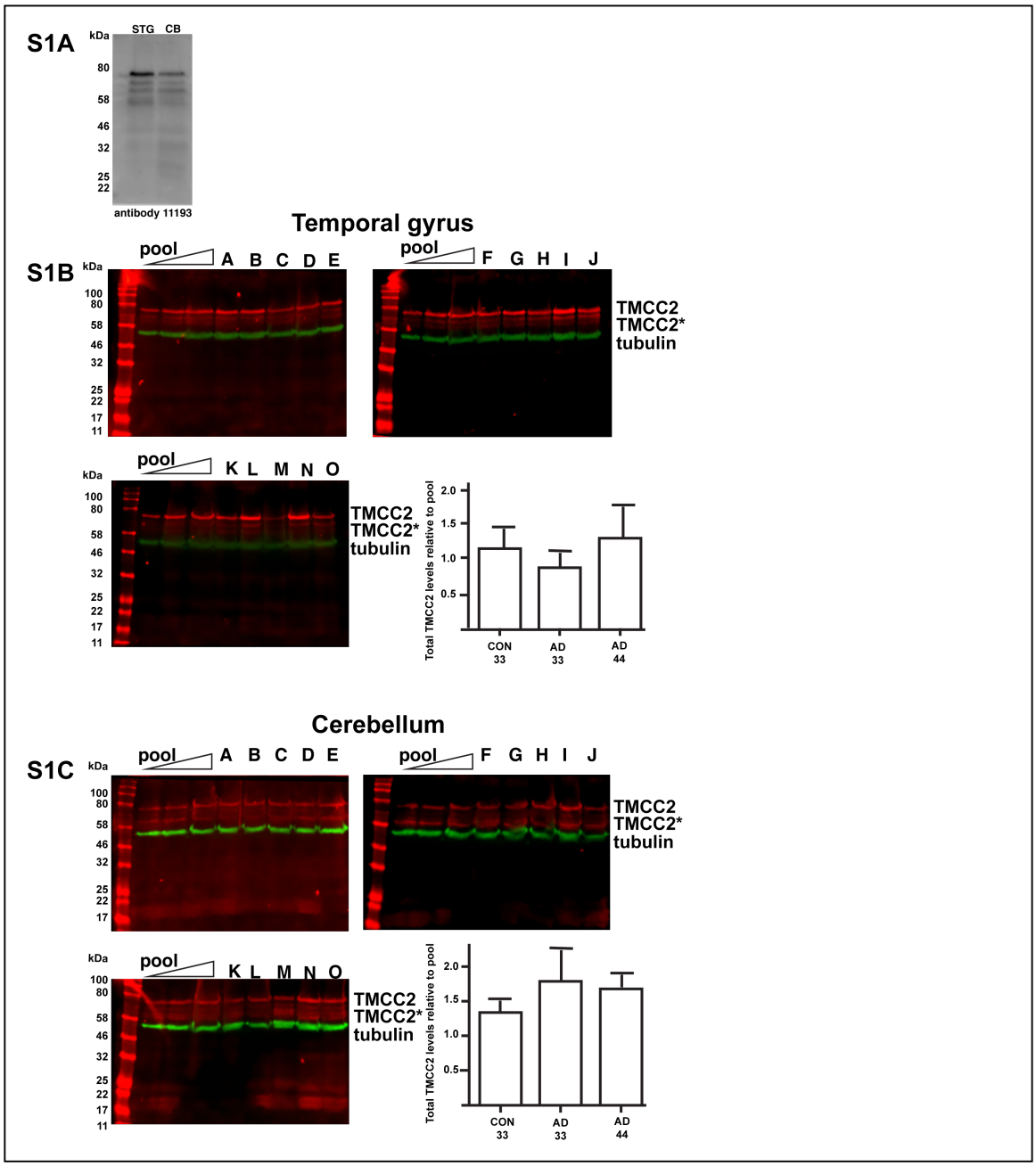
All procedures performed in studies involving human tissue were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical studies. Ethical approval was granted by Brains for Dementia Research City and East National Research Ethics Service committee (08/H0704/128+5) and specimens provided by the Manchester Brain Bank, Salford, UK. Cases were anonymized, but information was provided regarding APOE genotype, sex, age at death, and neuropathology (supplementary Table 1). Homogenates were prepared using an electrical homogenizer and SDS-containing buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.125 M Tris HCl, pH 6.8.) containing protease inhibitors (Complete, Boehringer Mannheim), followed by heating to 95°C for 5 minutes and centrifugation at 15,000 g for 10 minutes. We specifically note that efficient recovery of TMCC2 required brain homogenates to be incubated with SDS prior to centrifugation; non-ionic detergents such as Triton X-100 or NP40 did not efficiently solubilize TMCC2 from brain tissue.
Sections of 7μm were cut from formalin fixed paraffin embedded blocks from the same cases as used in immunoblot protocols.

Immunocytochemistry and western blots.

Rabbit anti-TMCC2 antibodies 94 and 11193 were raised against recombinant TMCC2 as previously described [4] and used at 1/300 for tissue staining in 10% normal goat serum, and 1/1000 for Western blots in 5% skim milk powder. SDS-PAGE gels were 10% acrylamide and loaded with 1 mg original tissue equivalent per lane. Data collection and quantification of band intensities for western blots was performed using the LI-COR Odyssey platform and statistical calculations used Graphpad Prism. Anti-Aβ antibody 4G8 was purchased from Chemicon (Temecula, CA, USA) and used at 1/300. Anti-phospho-tau antibody AT8 and anti-tubulin antibody DM1A were purchased from ThermoFisher (Loughborough, UK) and used at 1/200. Following dewaxing, for dual labeling with anti-TMCC2 (94) and AT8, antigen retrieval was sodium citrate pH 6.0, 100°C for 10 minutes; for dual labeling with anti-TMCC2 (94) and 4G8, C1/6.1 or methoxy-X04 this was preceded by 80% formic acid for 20 minutes. Tissue sections were blocked with 10% normal goat serum in PBS for 45 minutes and incubated with primary antibodies for 1 hour at room temperature. Secondary antibodies were Alexa-Fluor-488-conjugated goat anti-rabbit and Alexa-Fluor-546 or Alexa-Fluor-568-conjugated goat anti-mouse, from Invitrogen, and incubated for 1 hour at room temperature. Methoxy-X04 was used at 10 μM for 20 mins in PBS and autoflourescence quenched with Sudan black. Images were collected on a Zeiss Axio Imager.Z2 LSM 800 microscope and processed using Zeiss ZEN software and ImageJ.

Supplementary Figures

Figure S1. Alteration of TMCC2 according to brain region. S1A, western blot of human temporal cortex or cerebellum pools for TMCC2 using antibody 11193 confirming specificity of the TMCC2 and TMCC2* bands detected by antibody 94 shown in Fig. 1A. Pools are composed of equal quantities from each sample shown in S1B and S1C for temporal gyrus (STG) or cerebellum (CB). S1B, western blot of individual samples from temporal gyrus using antibody 94. S1C western blot of individual samples from cerebellum using antibody 94. Pools were loaded at a ratio of 0.5 to 1 to 2, with 1 representing approximately 1 mg of original tissue. Letters above lanes refer to cases described in Table S1. Bar charts show the relative levels of total TMCC2 for control APOE3 homozygotes, and AD cases homozygous for either APOE3 or APOE4 normalized between blots by reference to the band intensities of the pools; no significant association of APOE genotype or AD with total TMCC2 levels was found (P=0.63 for temporal gyrus and 0.24 for cerebellum, 1 way ANOVA).



				Specimen Characteristics				
Code	ID	Sex	Age	Clinical diagnosis	Pathological diagnosis 1	Pathological diagnosis 2	Braak stage	APOE genotype
A	BBN_3410	M	70	Dementia	Alzheimer's disease	very severe CAA	IV	33
B	BBN_3421	M	84	Cognitively normal	moderate SVD		0-I	33
C	BBN_3466	F	71	Alzheimer's disease	severe Alzheimer's disease		VI	44
D	BBN_3472	M	73	Alzheimer's disease	Alzheimer's disease	moderate SVD	VI	44
E	BBN_6080	F	81	Alzheimer's disease	Alzheimer's disease		IV-V	33
F	BBN_15257	M	77	FTD/PNFA	Alzheimer's disease		IV-V	33
G	BBN_15309	M	77	Vascular dementia	Alzheimer's disease		V	33
H	BBN_18819	F	89	Dementia	Alzheimer's disease	moderate SVD	V-VI	44
I	BBN_20005	M	85	Normal	age changes only	moderate SVD	0-I	33
J	BBN_21395	M	69	Alzheimer's disease	Alzheimer's disease	isocortical DLB	V-VI	44
K	BBN_24212	F	82	control	age changes only	mild SVD	II	33
L	BBN_25109	F	79	Dementia/AD	Alzheimer's disease		VI	44
M	BBN005.29168	M	90	Control	Normal for age	mild SVD	I-II	33
N	BBN005.29398	M	84	Control	Normal ageing	mild to moderate SVD	II	33
O	BBN005.30048	M	80	Vascular dementia	Alzheimer's disease		V	33

Supplementary Table S1. Case characteristics. AD, Alzheimer’s disease; SVD, small vessel disease; CAA, cerebral amyloid angiopathy; FTD, frontotemporal dementia; PNFA, progressive non fluent aphasia; DLB, dementia with Lewy bodies.