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Authors
Robinson, John L
Molina-Porcel, Laura
Corrada, Maria M
et al.

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Perforant path synaptic loss correlates with cognitive impairment and Alzheimer's disease in the oldest-old

John L. Robinson,[1] Laura Molina-Porcel,[1] Maria M. Corrada,[2] Kevin Raible,[1] Edward B. Lee,[1] Virginia M.-Y. Lee,[1] Claudia H. Kawas[3] and John Q. Trojanowski[1]

1 Centre for Neurodegenerative Disease Research, Institute on Aging and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA
2 Department of Neurology, and Institute for Memory Impairments and Neurological Disorders, University of California at Irvine, Irvine, CA, USA
3 Department of Neurology, Department of Neurobiology and Behaviour, and Institute for Memory Impairments and Neurological Disorders, University of California at Irvine, Irvine, CA, USA

Correspondence to: John Q. Trojanowski, M.D., Ph.D.,
Centre for Neurodegenerative Disease Research and Institute on Aging,
Department of Pathology and Laboratory Medicine,
University of Pennsylvania School of Medicine, HUP,
Maloney 3rd Floor, 36th and Spruce Streets, Philadelphia,
PA 19104-4283, USA
E-mail: trojanow@mail.med.upenn.edu

Alzheimer's disease, which is defined pathologically by abundant amyloid plaques and neurofibrillary tangles concurrent with synaptic and neuronal loss, is the most common underlying cause of dementia in the elderly. Among the oldest-old, those aged 90 and older, other ageing-related brain pathologies are prevalent in addition to Alzheimer's disease, including cerebrovascular disease and hippocampal sclerosis. Although definite Alzheimer's disease pathology can distinguish dementia from normal individuals, the pathologies underlying cognitive impairment, especially in the oldest-old, remain poorly understood. We therefore conducted studies to determine the relative contributions of Alzheimer's disease pathology, cerebrovascular disease, hippocampal sclerosis and the altered expression of three synaptic proteins to cognitive status and global cognitive function. Relative immunohistochemistry intensity measures were obtained for synaptophysin, Synaptic vesicle transporter Sv2 (now known as SV2A) and Vesicular glutamate transporter 1 in the outer molecular layer of the hippocampal dentate gyrus on the first 157 participants of 'The 90+ Study' who came to autopsy, including participants with dementia (*n* = 84), those with cognitive impairment but no dementia (*n* = 37) and those with normal cognition (*n* = 36). Thal phase, Braak stage, cerebrovascular disease, hippocampal sclerosis and Pathological 43-kDa transactive response sequence DNA-binding protein (TDP-43) were also analysed. All measures were obtained blind to cognitive diagnosis. Global cognition was tested by the Mini-Mental State Examinaton. Logistic regression analysis explored the association between the pathological measures and the odds of being in the different cognitive groups whereas multiple regression analyses explored the association between pathological measures and global cognition scores. No measure clearly distinguished the control and cognitive impairment groups. Comparing the cognitive impairment and dementia groups, synaptophysin and SV2 were reduced, whereas Braak stage, TDP-43 and hippocampal sclerosis frequency increased. Thal phase and VGLUT1 did not distinguish the cognitive impairment and dementia groups. All measures distinguished the dementia and control groups and all markers associated with the cognitive test scores. When all markers were analysed simultaneously, a reduction in synaptophysin, a high Braak stage and the presence of TDP-43 and hippocampal sclerosis associated with global cognitive function. These findings suggest that tangle pathology, hippocampal sclerosis, TDP-43 and perforant pathway synaptic loss are the major contributors to dementia in the oldest-old. Although an increase in plaque pathology and glutamatergic synaptic loss may be early events associated

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with cognitive impairment, we conclude that those with cognitive impairment, but no dementia, are indistinguishable from cognitively normal subjects based on the measures reported here.

Keywords: Alzheimer's disease; Braak stage; Thal phase; synaptic loss; oldest-old; cognitive impairment

Abbreviations: CIND = cognitively impaired no dementia; MMSE = Mini-Mental State Examination; RIR = relative immunointensity ratio

Introduction

Along with tangles and plaques, Alzheimer's disease is characterized by the loss of neurons and their synapses. Indeed, significant synapse loss has been documented in all brain regions affected by Alzheimer's disease pathology (DeKosky *et al*., 1996) and reductions in the number of presynaptic and synaptic proteins may be the most consistent progression marker of Alzheimer's disease as the burden of plaques and tangles grows and neurons continue to degenerate (Beeri *et al*., 2012). For example, significant reductions in synaptophysin and VGLUT1 were found in both the parietal and occipital cortices of Alzheimer's disease brains in one study (Kirvell *et al*., 2006) and our group previously reported a decrease in synaptophysin in the frontal cortex of dementia patients (Head *et al*., 2009). Nonetheless, the relationship between anatomy, synaptic proteins and Alzheimer's disease pathology is complex (Honer, 2003). In our previous study, individuals with cognitive impairment, but no dementia (CIND) had increases in synaptophysin whereas others have reported a significant decrease in the hippocampal levels of synaptophysin, even in CIND individuals (Sze *et al*., 2000).

Because it has long been known that changes in the perforant pathway are associated with memory impairment (Hyman *et al*., 1986; Senut *et al*., 1991), we hypothesized that perforant pathway synaptic changes may be one of the major correlates of cognitive impairment in Alzheimer's disease. The perforant pathway arises from neurons in layers 2 and 3 of the entorhinal cortex and projects into the outer molecular layer of the hippocampal dentate gyrus. Importantly, neurons and synapses in the inner molecular layer remain unaffected by perforant pathway loss in the outer molecular layer. There are several markers of synaptic health that are reliably detected by immunohistochemistry in sections of CNS tissues including synaptophysin, SV2 and VGLUT1.

Synaptophysin is involved in multiple, important aspects of synaptic vesicle exo- and endocytosis (Valtorta *et al*., 2004). SV2, besides being a ubiquitous presynaptic protein, is the binding site for the antiepileptic drug levetiracetam, a drug shown to improve cognition in CIND individuals (Bakker *et al*., 2012). VGLUT1 mediates the accumulation of glutamate—the major excitatory neurotransmitter—into secretory vesicles in the neocortex and hippocampus (El Mestikawy *et al*., 2011).

Individuals in the 'The 90+ Study', a population-based cohort of nonagenarians, have a high prevalence of cognitive impairment (Peltz *et al*., 2012) allowing for the assessment at autopsy of a significant number of CIND individuals. This investigation is a cross-sectional clinical-pathological correlation study involving the first 157 individuals to come to autopsy. For our analysis, we compared measures of perforant pathway synaptic health across each cognitive group. Since the prevalence of severe Alzheimer's disease pathology is reduced in nonagenarians, whereas the proportion with hippocampal sclerosis and cerebrovascular disease pathology increases (Nelson *et al*., 2011*b*), we also measured the underlying pathologies affecting these individuals, specifically their Braak stage, Thal phase, and the presence of hippocampal sclerosis and cerebrovascular disease. Our aim was to understand both the synaptic and pathological changes underlying cognitive impairment and dementia.

Materials and methods

Study participants were the first 157 individuals to come to autopsy from the 90+ Study, a longitudinal population-based study of ageing and dementia in people aged 90 and older who are survivors of the Leisure World Cohort Study (Corrada *et al*., 2012). Briefly, individuals live at home as well as in institutions, and represent the full spectrum of health and cognitive abilities. All 90+ Study participants had evaluations every 6 months including a neurological examination by a trained physician or nurse practitioner and a full neuropsychological battery that included the Mini-Mental State Examination (MMSE). Relevant medical history, medication use, and demographic information were obtained from the participants or their informants. Medical records, including brain imaging evaluations were obtained from the participant's physicians. Information about cognitive (Clark and Ewbank, 1996) and functional abilities (Pfeffer *et al*., 1982) were obtained from informants in frequent contact with the participants. To inquire about the onset of cognitive problems, the Dementia Questionnaire (Silverman *et al*., 1986; Kawas *et al*., 1994) interview was conducted over the phone with informants of participants with evidence of cognitive impairment. Shortly after death, the Dementia Questionnaire was done with the decedent's informant to inquire about the participant's condition since the last evaluation. The Institutional Review Board of the University of California, Irvine, approved all procedures and all participants or their surrogates gave written informed consent.

Determination of cognitive status

After a participant's death, all available information was reviewed and discussed during a multidisciplinary consensus diagnostic conference led by 'The 90+ Study' principal investigator (C.K.). Participants were classified as normal, CIND, or as having dementia. Dementia diagnosis was established using Diagnostic and Statistical Manual of Mental Disorders 4th Edition criteria (American Psychiatric Association *et al*., 1994). CIND is defined by initial cognitive impairments such as deficits in episodic memory (Albert *et al*., 2001), executive dysfunction (Chen *et al*., 2001), naming difficulties or other aphasias (Saxton *et al*., 2004). Participants were classified as CIND if they showed cognitive or functional deficits that were not severe enough to meet criteria for dementia. All cognitive diagnoses were made blinded to pathological evaluations.

Neuropathology

All autopsies were performed at the University of California, Irvine. After weighing the whole brain and gross inspection, one hemisphere was dissected as previously described (Berlau *et al*., 2009). Six-micrometre thick, coronal sections of mid-frontal cortex superior temporal cortex, anterior hippocampus, amygdala, substantia nigra and medulla oblongata were cut. All histological staining, immunohistochemistry and microscopic analyses were performed in the Centre for Neurodegenerative Disease Research (CNDR) at the University of Pennsylvania as described (Robinson *et al*., 2011). Briefly, sections were subjected to immunohistochemistry using the avidin-biotin complex detection method (VECTASTAIN® ABC kit; Vector Laboratories) with ImmPACT™ diaminobenzidine peroxidase substrate (Vector Laboratories) as the chromogen using monoclonal antibodies to phosphorylated tau (mouse PHF1; 1:1K, gift of Dr Peter Davies, Manhasset, NY), β-amyloid (mouse NAB228; 1:15K; generated in CNDR), TARDBP (rat 409/410; 1:500; gift of Dr Manuela Neumann, Zurich, Switzerland), SV2 (mouse SV2; 1:20K; DSH Iowa), synaptophysin (mouse MAB368; 1:1K; Millipore) and VGLUT1 (Guinea pig VGLUT1; 1:7.5K; SYSY).

Topographical Braak staging (stages I–VI) was assigned from PHF1 stained slides ($n = 157$) (Braak *et al*., 2006). Thal phases were determined from NAB228 stained hippocampal slides: phase 0–1, 2, 3 and 4 ($n = 150$) (Thal *et al*., 2006). TARDBP inclusions and neurites were determined from 409/410 stained hippocampal slides: presence/absence.

The assessment of cerebrovascular disease pathology ($n = 108$) and hippocampal sclerosis ($n = 155$) was determined from Harris haematoxylin and eosin stained mid-frontal cortex, superior temporal cortex, hippocampus, amygdala, substantia nigra and medulla sections. Hippocampal sclerosis was assessed as follows: 0 for no gliosis or neuronal loss; 1+ for mild gliosis or neuronal loss in the CA1 or subiculum; 2+ for moderate or severe gliosis and neuronal loss consistent with definite hippocampal sclerosis. Cerebrovascular lesions such as infarcts, micro-infarcts or micro-bleeds along with cerebral amyloid angiopathy and hippocampal sclerosis were used to generate cerebrovascular disease pathology scores following a simplified staging of Jellinger and Attems as follows: 0 for cases without major infarcts, hippocampal sclerosis, cerebral amyloid angiopathy or other lesions; 1+ for minimal vascular pathology cases with mild to moderate cerebral amyloid angiopathy and/or 1–2 small lacunes; 2+ for moderate vascular pathology cases with severe cerebral amyloid angiopathy and/or hippocampal sclerosis and/or major infarcts (Jellinger and Attems, 2003).

Synaptic relative immunointensity ratios (RIRs) were obtained by the following process. A preliminary study of synaptic and presynaptic proteins was done including antibodies to synaptophysin, SV2, VGLUT1, synataxin 1, VAMP2, synapsin 1, synaptotagmin 1, dynamin, VGAT, PSD95 and GLUR1; obvious outer molecular layer synaptic loss was observed on slides obtained from patients with Alzheimer's disease when stained with antibodies to synaptophysin, SV2 and VGLUT1. For this study, hippocampal slides were stained for synaptophysin ($n = 149$), SV2 ($n = 151$) or VGLUT1 ($n = 152$). Sections were scanned (Nikon DS-Fi2 camera, gain 1.2, exposure 15 ms) at $\times 10$ on a Nikon Eclipse TE2000 microscope using NIS Elements software (Nikon Instruments, Inc.). ImageJ 1.47t (National Institutes of Health) was used for analysis. Mean pixel intensities of the inner- and outer molecular layer of the dentate gyrus just under CA1 were captured (Fig. 1). Raw values on an 8-bit scale (range of 45–185) were normalized to a blank area for each slide (median value 41). Synaptic RIRs of the outer/inner molecular layer were calculated from (outer molecular layer − blank) / (inner molecular layer − blank) for each case. To verify the repeatability of our measurements, 10 random cases were stained twice by the synaptic antibodies and synaptic RIRs were obtained independently by two researchers (L.M.P., J.L.R.). A *K* of 0.987 was obtained (Type C intraclass correlation coefficient).

All pathological diagnoses were done blinded to clinical diagnosis.

Statistical analysis

We compared characteristics of the three cognitive groups: normal, CIND and dementia using χ^2 tests for categorical variables and ANOVA for continuous variables. We used multinomial logistic regression models to determine the association between each individual neuropathological or synaptic protein measure and cognitive diagnosis. We report the odds of being in the CIND group compared to the normal group, the odds of being in the dementia group compared to the CIND group, and the odds of being in the dementia group compared to the normal group. Neuropathological and synaptic protein measures were analysed as continuous variables in the logistic regression analyses. Separate regression models were used for each neuropathological and synaptic protein measure. We also explored the association between the individual neuropathological or synaptic measures and the MMSE, a measure of global cognition, using multiple linear regression analyses. Finally, we analysed the relative contribution of the different pathological measures to global cognitive scores, by including all synaptic and pathological measures in a multiple regression model. All regression models were adjusted for age at death and gender and all analyses were performed using SAS version 9.3 (SAS Institute Inc.).

Results

Subject characteristics

Of the 157 participants of this study, 36 had normal cognition, 37 were diagnosed with CIND and the majority ($n = 84$) had dementia (Table 1). The most frequent clinical diagnosis of the participants with dementia was Alzheimer's disease alone (65%) or in combination with other dementias (21%) followed by vascular dementia (7%) and dementia with Lewy bodies (6%).

Participants had an average age at death of 98 years (range: 94–101 years), were mostly female (71%), and highly educated (71% had at least a college education). MMSE scores were available for the majority of individuals within 1 year before death (Table 1). The overall frequency of the *APOE* alleles were 14% for the e2 allele and 22% for the e4 allele. While there was an increase in the frequency of the e4 allele in the dementia group (27%) compared to the normal group (21%), this non-significant increase is consistent with previous work where we found that the e4 allele no longer plays a role in dementia and mortality at very old ages (Corrada *et al*., 2013). Brain weight was non-significantly lower in the dementia group.

Alzheimer's disease pathology

In the normal group, Braak stage (median III; mean 3.3) and Thal phase (median 1; mean 1.9) scores indicate that Alzheimer's disease pathology was present in at least mild to moderate amounts (Fig. 2D and E). The CIND group presented with similar Braak

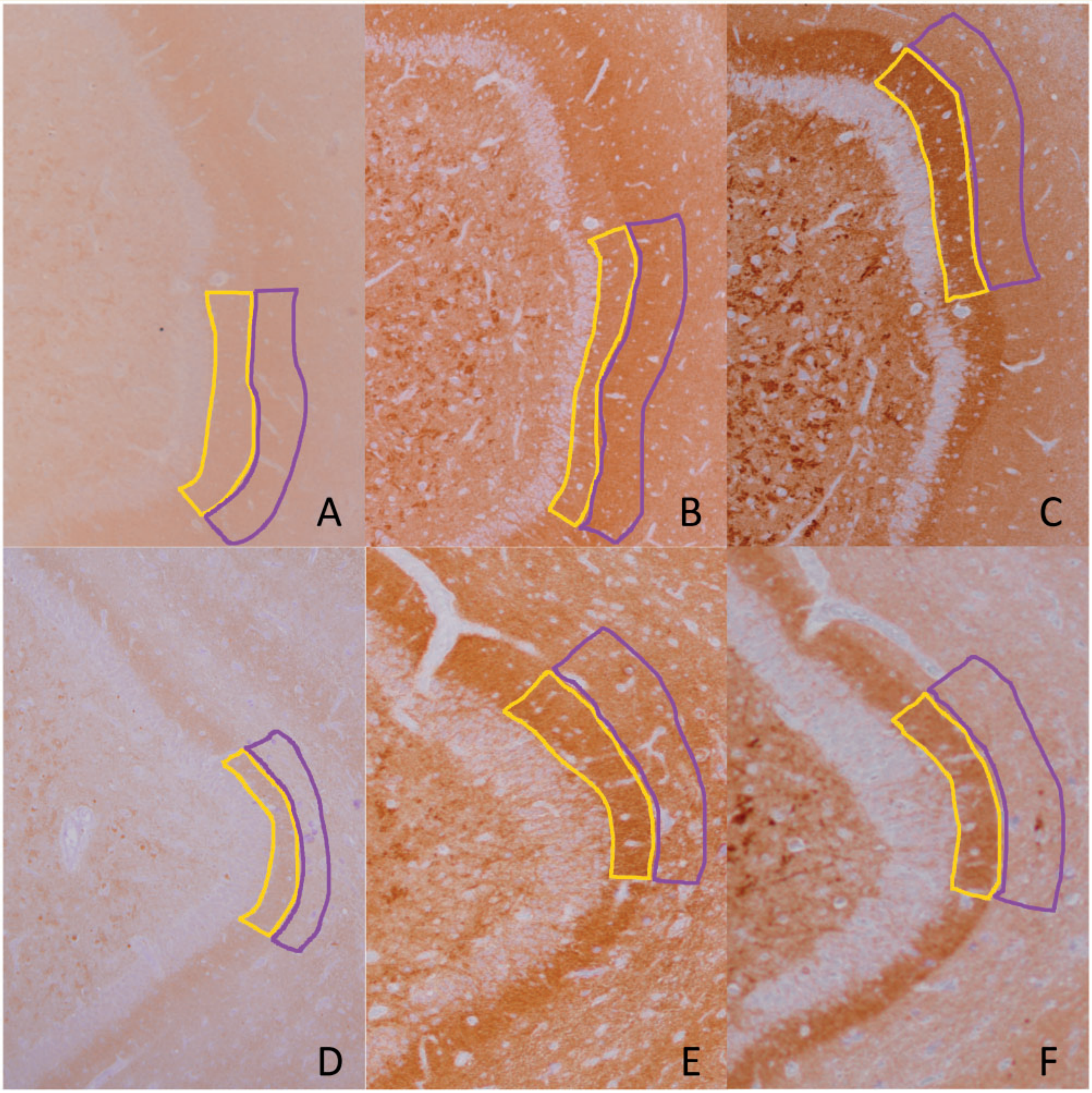

Figure 1 Representative images of synaptic protein immunoreactivity. Synaptic marker immunohistochemistry from two cases shows distinct staining of the inner molecular layer (highlighted in yellow) and the outer molecular (highlighted in purple) of the hippocampus. (**A–C**) The relatively healthy outer molecular layer: (**A**) synaptophysin, (**B**) SV2 and (**C**) VGLUT1 with synaptic ratios 1.19, 1.13 and 0.78, respectively (note that VGLUT1 is consistently lower than the other two). In contrast, (**D–F**) represents an individual with severely reduced outer molecular layer, whose inner molecular layer is preserved: (**D**) synaptophysin, (**E**) SV2 and (**F**) VGLUT1 with synaptic ratios 0.53, 0.74 and 0.56, respectively.

stage (median III; mean 3.4) and Thal phase (median 3; mean 2.3) scores, while Braak stage (median V; mean 4.3) and Thal phase (median 3; mean 2.6) were more moderate to severe in the dementia group. Higher Braak stages were significantly associated with higher odds of being in the dementia versus normal group [odds ratio (OR) = 1.68, $P = 0.001$] and of being in the dementia versus CIND group (OR = 1.52, $P = 0.02$) (Table 2). Higher Thal phases were associated with higher odds of being in the dementia versus the normal group (OR = 1.64, $P = 0.011$) but did not distinguish between the dementia and CIND groups ($P > 0.15$). Neither Alzheimer's disease marker distinguished between the normal and CIND groups.

Higher levels of Alzheimer's disease plaque and tangle burdens were significantly associated with lower MMSE scores (Thal phase,

Table 1 Characteristics of The 90+ Study participants

Characteristic	All subjects (*n* = 157)	Normal (*n* = 36)	CIND (*n* = 37)	Dementia (*n* = 84)
	Mean (SD)			
Age at death	98.0 (3.6)	97.8 (2.5)	98.4 (4.0)	97.7 (3.7)
Last MMSE score[a]	18.1 (10.1)	27.8 (1.6)	23.7 (5.4)	11.2 (8.6)
MMSE interval to death (months)	7.5 (7.7)	5.9 (3.3)	6.5 (4.5)	8.6 (9.8)
Brain weight (g)	1127 (121)	1179 (104)	1123 (125)	1104 (121)
	***n* (%)**			
APOE E4[b]				
0 alleles	119 (78)	27 (79)	33 (89)	59 (73)
⩾ 1 alleles	33 (22)	7 (21)	4 (11)	22 (27)
APOE E2				
0 alleles	131 (86)	29 (85)	31 (84)	71 (88)
⩾ 1 alleles	21 (14)	5 (15)	6 (16)	10 (12)
Gender				
Male	45 (29)	15 (42)	10 (27)	20 (24)
Female	112 (71)	21 (58)	27 (73)	64 (76)
Education[c]				
⩽ High school	45 (29)	7 (19)	8 (22)	30 (36)
Any college	73 (47)	16 (44)	20 (54)	37 (45)
Any graduate school	38 (24)	13 (36)	9 (24)	16 (19)
Normal cognition	36 (23)	36 (100)		
CIND	37 (24)			
Memory impairment			13 (35)	
Executive impairment			12 (32)	
Other impairment[d]			12 (32)	
Dementia	84 (54)			
Alzheimer's disease only				55 (65)
Alzheimer's disease plus[e]				18 (21)
Vascular dementia				6 (7)
Other dementia				5 (6)

[a]Excludes four participants with missing MMSE score.
[b]Excludes five participants with unknown ApoE allele status.
[c]Excludes one participant with unknown degree of education.
[d]Includes impairment in domains other than memory or executive function.
[e]Includes mixed Alzheimer's disease/vascular dementia, Alzheimer's disease/other and Alzheimer's disease with dementia with Lewy bodies.

$P = 0.02$; Braak stage, $P < 0.001$). The greatest decreases on the cognitive tests occurred with the highest plaque and tangle burdens. The mean MMSE score was significantly lower in Thal phase 4 compared to Thal phases 2 and earlier ($P < 0.01$) and even to Thal phase 3 ($P < 0.05$). For Braak stage, the mean MMSE score was significantly lower in stage VI compared to all the other Braak stages ($P < 0.001$), and in stage V compared to stages III and IV ($P < 0.05$).

Hippocampal sclerosis and cerebrovascular disease

As the prevalence of hippocampal sclerosis and cerebrovascular disease pathology increases with age, we investigated what proportion of individuals had these lesions in our cohort. Hippocampal sclerosis was found in 15% of all individuals, but in only one individual without dementia. The presence of hippocampal sclerosis in 28% of those with dementia (23/82) allowed the measure to significantly distinguish the dementia group from both the normal ($P = 0.003$) and CIND ($P = 0.002$) groups (Table 2).

Cerebrovascular lesions and cerebral amyloid angiopathy were present in modest amounts in The 90+ Study. Cerebral amyloid angiopathy was found in 52% (56/108) of the cases whereas cerebrovascular disease lesions such as infarcts, micro-infarcts or micro-bleeds were rarer (10%; 11/108). Althogether, 63% of all individuals had some level of cerebrovascular disease pathology, including 45% of both the normal and CIND groups and 74% of the dementia group. Although the prevalence of cerebrovascular disease was not different between the normal and CIND groups (Fig. 2F), people with higher levels were more likely to be in the dementia group compared to both the normal and CIND groups (both $P = 0.009$) (Table 2).

TDP-43

TDP-43 is a common co-morbidity and may associate with a more rapid cognitive decline in the ageing brain (Wilson *et al*., 2013). In

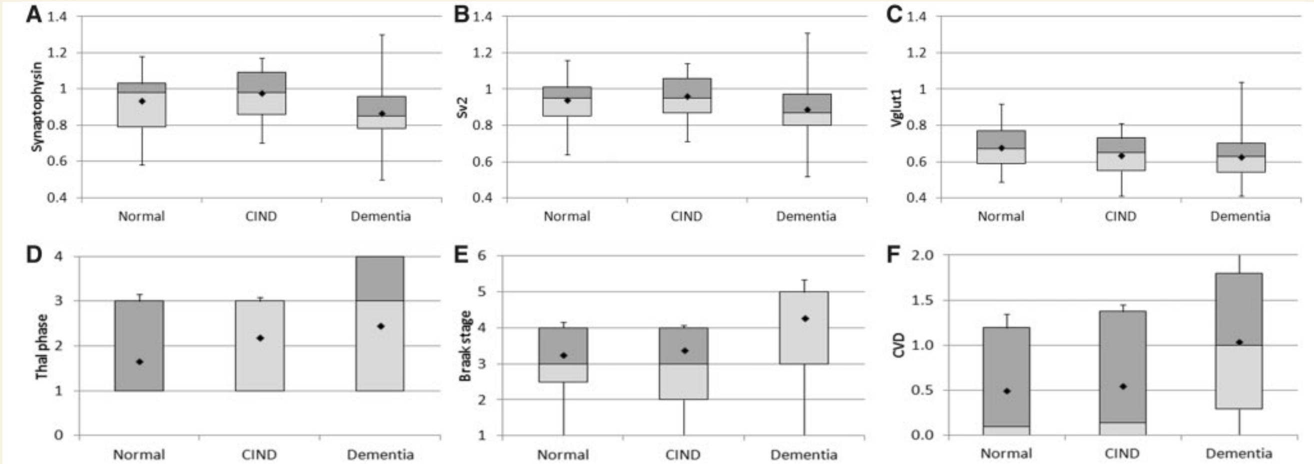

Figure 2 Synaptic intensity and neuropathology measures by cognitive group. Synaptic ratios (**A–C**) and neuropathology measures (**D–F**) in the oldest-old. All three synaptic RIRs were reduced in dementia compared to normal with (**A**) synaptophysin and (**B**) SV2 and significantly different between the three cognitive groups, however, (**C**) VGLUT1 did not distinguish between CIND and dementia. No synaptic RIR distinguished normal from CIND. (**D**) Thal phase and scores were all increased in dementia compared to normal with (**D**) similar Thal phases reported for CIND and dementia, whereas (**E**) Braak stage and (**F**) cerebrovascular disease (CVD) pathologies, including hippocampal sclerosis, were significantly different between the three cognitive groups. Each measure is graphed with the first and third quartiles boxed with the median band dividing the box into darker upper and lighter lower ranges; a whisker line extends to the minimums and maximums; shaded diamonds represent the means.

Table 2 Association between markers and cognitive groups

Measure	Wald test *P*-value	CIND versus Normal OR (95% CI) *P*-value	Dementia versus CIND OR (95% CI) *P*-value	Dementia versus Normal OR (95% CI) *P*-value
Synaptophysin	0.002	5.59 (0.20–159.45) 0.314	0.01 (<0.01–0.11) **0.001**	0.03 (<0.01–0.59) **0.021**
SV2	0.017	2.85 (0.10–84.75) 0.546	0.02 (<0.01–0.35) **0.008**	0.05 (<0.01–0.93) **0.045**
VGLUT1	0.099	0.04 (<0.01–2.41) 0.124	0.56 (0.02–18.84) 0.747	0.02 (<0.01–0.76) **0.034**
Braak stage	0.001	1.06 (0.75–1.50) 0.740	1.52 (1.07–2.16) **0.020**	1.68 (1.24–2.30) **0.001**
Thal phase	0.037	1.37 (0.89–2.11) 0.158	1.20 (0.84–1.71) 0.318	1.64 (1.12–2.38) **0.011**
Hippocampal sclerosis	<0.001	0.94 (0.36–2.49) 0.906	3.32 (1.56–7.05) **0.002**	3.13 (1.48–6.64) **0.003**
Cerebrovascular disease	0.004	0.94 (0.35–2.55) 0.900	2.99 (1.31–6.83) **0.009**	2.81 (1.29–6.13) **0.009**
TDP-43	0.008	0.90 (0.20–4.05) 0.893	4.32 (1.34–13.95) **0.014**	3.89 (1.23–12.36) **0.021**

Odds ratios and 95% CIs were generated from multinomial logistic regression models where the dependent (outcome) variable was cognitive group (normal, CIND, or dementia) and the independent variables were the neuropathological markers as continuous variables, except TARDBP which was a binary variable. *P*-values <0.05 are in bold. Age at death and gender were included as covariates and each neuropathological measure was analysed in a separate model. The overall *P*-value corresponds to the type 3 analyses and tests whether the neuropathological marker (independent variable) is significantly associated with the cognitive group (outcome variable). Odds ratios are per unit. *n* for each group = 149 (synaptophysin), 151 (SV2), 152 (VGLUT1), 157 (Braak stage), 150 (Thal phase), 155 (hippocampal sclerosis, 108 (cerebrovascular disease) and 146 (TARDBP).

our cohort, TDP-43 pathology was present in the hippocampus of 24% (35/146) of the cases. While a small number of both normal (11%, 4/35) and CIND (12%, 4/33) individuals had pathology, TARDBP inclusions affected a more substantial portion of the dementia group (35%, 27/78). As in our earlier study (Robinson *et al*., 2011), TARDBP was almost exclusively in individuals with hippocampal sclerosis. 77% (17/22) of the cases with hippocampal sclerosis also had TARDBP inclusions. While 15% (18/124) of individuals without hippocampal sclerosis also had TARDBP pathology, the numbers were too low to analyse the significance of the TARDBP pathology after controlling for hippocampal sclerosis (data not shown).

Synaptic health

As described above, to measure the synaptic levels of the perforant pathway, synaptic RIRs were generated by measuring the intensity of synaptophysin, SV2, and VGLUT1 by immunohistochemistry in the outer molecular layer of the hippocampus relative to the inner molecular layer (Fig. 1). Higher RIRs represent a relatively healthy outer layer in relation to the inner layer, whereas lower RIRs correspond to synaptic loss in the outer layer (Fig, 2A–C). In the normal group, synaptophysin (mean 0.94) and SV2 (mean 0.94) had RIRs close to 1.00, whereas the glutamate-specific VGLUT1 RIR was lower (mean 0.67). Similar values were obtained in the CIND group: synaptophysin (mean 0.98), SV2 (mean 0.96) and VGLUT1 (mean 0.64). In the dementia group, these values were mildly lower: synaptophysin (mean 0.87), SV2 (mean 0.89) and VGLUT1 (mean 0.63).

All three synaptic RIRs distinguished between the dementia and normal groups with higher RIRs associated with lower odds of being in the dementia group ($P < 0.05$) (Table 2). The synaptophysin and SV2 RIRs also distinguished between the dementia and CIND groups (synaptophysin, $P < 0.001$; SV2, $P = 0.008$), whereas the VGLUT1 RIR did not ($P = 0.747$). No RIR distinguished between the CIND and normal groups (all $P > 0.12$). We also examined the association between synaptic RIRs and global cognitive scores and found that for all three synaptic markers, higher RIR values were significantly associated with higher MMSE scores (synaptophysin, $P < 0.001$; SV2, $P < 0.001$, VGLUT1, $P < 0.05$).

Multiple pathologies

Pearson correlation coefficients showed significant correlations between the three synaptic RIRs (VGLUT1 and SV2, corr = 0.37, $P < 0.001$; VGLUT1 and synaptophysin, corr = 0.45, $P < 0.001$; SV2 and synaptophysin, corr = 0.50, $P < 0.001$). Given the correlation between synaptic markers, we wanted to explore the independent contribution of each with respect to global cognitive scores. In a multiple regression model that included all three synaptic markers, we found that higher RIRs for both synaptophysin (beta = 20.3, $P = 0.002$) and SV2 (beta = 13.4, $P = 0.03$) remained significantly associated with better MMSE scores.

Post hoc analysis revealed that the SV2 RIR was significantly lower at Braak stage VI ($P < 0.001$) and the VGLUT1 RIR was significantly lower in individuals with hippocampal sclerosis ($P = 0.024$). To estimate the independent contribution of all the different measures to global cognitive scores, we included all three synaptic RIRs, the Alzheimer's disease pathology scores, and hippocampal sclerosis in a multiple regression analysis. In this model, the synaptophysin ratio, Braak stage, TARDBP and hippocampal sclerosis all remained significantly associated with MMSE scores (P-values: < 0.05, 0.001, <0.01 and 0.001, respectively).

Memory impairment only subjects

The primary impairment in 13 of the CIND individuals was memory (Table 1). As those with executive impairment may have dysfunction related more to a frontotemporal lobar degeneration rather than Alzheimer's disease and those with 'other' impairment cannot be well defined, it's possible that the 13 with memory impairment are more likely to be on the continuum between normal cognition and Alzheimer's disease. To test this hypothesis, we compared the RIRs and our pathological markers in the 'memory impairment only' CIND subjects to the normal and dementia subjects to see if our results had more significance. No new associations were found (data not shown), suggesting that the memory impairment subjects weren't more likely to lead to the intermediate level of Alzheimer's disease apparent in the dementia group than the other CIND individuals. Similar subgroup analysis with the executive impairment and 'other' impairment CIND individuals also did not reveal any new associations that the comparisons with the group as a whole hadn't already shown (data not shown).

Discussion

We hypothesized that perforant pathway synaptic loss may be one of the early correlates of cognitive impairment in The 90+ Study, but this was not the case. None of the measures distinguished the normal and CIND groups and almost all the measures distinguished the CIND and dementia groups from the cognitively normal individuals. This implies that synaptic loss is tightly linked to pathology associated with dementia in the oldest-old.

This is not the case in younger age groups where it is hypothesized that plaques and neurofibrillary tangles accumulate years, even decades, before cognitive impairment and where pathology accumulates progressively (Sperling *et al*., 2011). Perhaps nonagenarians represent individuals with significant cognitive reserve? Although their brains may be exposed to the same pathological insults during progressive cognitive impairment as younger cohorts, they are able to minimize any synaptic loss and accumulations of pathology. At the very least, our data are consistent with an acceleration of the pathological cascade implied by other studies.

In this autopsy study of a large cohort of CIND individuals, we sought to elucidate which pathologies among those examined here associate with normal ageing, which associate with dementia and which, if any, associate with cognitive impairment. We review all of our findings below.

First, cognitively normal nonagenarians in The 90+ Study have a median Braak stage of III and a Thal phase of 1 consistent with predictable, age-dependent deposition of Alzheimer's disease pathology (Braak *et al*., 2011). Of course, many pathologies besides Alzheimer's disease affect the ageing brain including other neurodegenerative diseases, cerebrovascular disease and hippocampal sclerosis among others. In this study, we did not examine the role of multiple neurodegenerative diseases, but we previously reported that α-synuclein and TARDBP pathology affect $<20\%$ of cognitively normal individuals in this age category and then

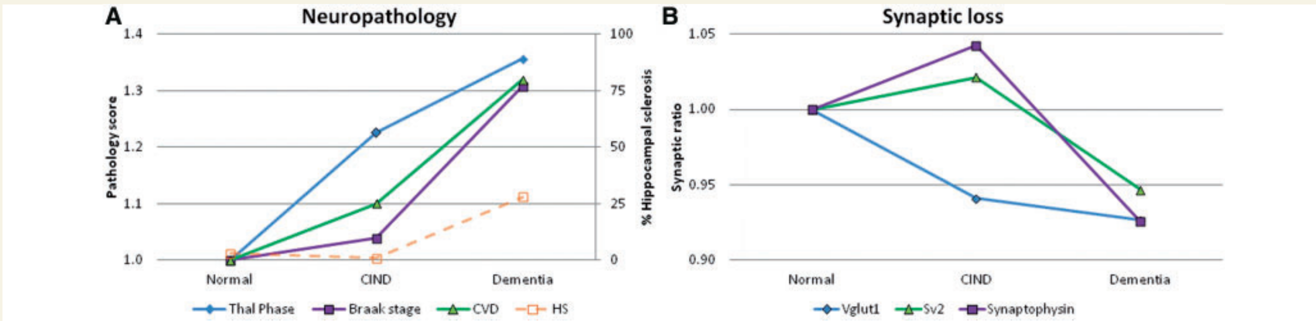

Figure 3 A model of changes associated with cognitive impairment and dementia in the oldest-old. Neuropathology and synaptic measures are associated with cognitive impairment in 'The 90+ Study'. (**A**) Mild to moderate Alzheimer's disease pathology already exists in the oldest-old for cognitively normal individuals and the Thal phase may increase with cognitive impairment. With dementia, all markers increase significantly, especially Braak stage, cerebrovascular disease (CVD) pathology and hippocampal sclerosis (HS). (**B**) Glutamatergic specific synaptic loss (VGLUT1) in the perforant pathology may occur during cognitive impairment, while significant synaptic loss occurs with dementia. All values are relative to the normal group's means normalized to 1.0 (see Fig. 1), except cerebrovascular disease which factors out the contribution of hippocampal sclerosis before normalizing; the hippocampal sclerosis percentages are graphed separately on the right axis.

primarily as co-morbidities (Robinson *et al*., 2011). Cerebrovascular disease, on the other hand, was common, affecting 45% of the group while hippocampal sclerosis was almost non-existent ($n = 1$).

Against this background level of multiple age-related pathologies in cognitively normal individuals, we can analyse the increases in Alzheimer's disease burden, cerebrovascular disease and hippocampal sclerosis associated with dementia (Fig. 3A). As 90+ individuals have a moderate burden of age-related tangles and plaques and a particularly high prevalence of dementia (Corrada *et al*., 2008), it is not surprising that Alzheimer's disease pathology is the most common underlying contributor to dementia in this group (Hebert *et al*., 2013). For individuals with dementia, the median Braak stage is V and the Thal phase of 3. Compared with the normal group, these are substantial increases consistent with an intermediate burden of Alzheimer's disease neuropathological change (Montine *et al*., 2012). Along with Alzheimer's disease, the prevalence of cerebrovascular disease—not including hippocampal sclerosis—increased to 74% of individuals in the dementia group. Additionally, dementia was associated with the appearance of hippocampal sclerosis in a substantial minority (28%). Similarly, and primarily comorbidly, TARDBP also affected a large minority (35%). Both the cerebrovascular disease and hippocampal sclerosis percentages are consistent with reported frequencies of these pathologies in this age-group (Giannakopoulos *et al*., 1997; Nelson *et al*., 2011*a*).

The changes in all three of the synaptic markers studied here are consistent with the interpretation that significant perforant pathway loss occurs with dementia in the 90+ subjects (Fig. 3B). This is consistent with our previous work involving 32 individuals in The 90+ Study where neocortical synaptophysin protein levels were significantly decreased with dementia (Head *et al*., 2009). In our multiple logistic regression analysis, both the synaptophysin RIR and Braak stage tightly associated with cognitive scores. Concurrently, *post hoc* analysis revealed that the SV2 RIR was significantly lower at Braak stage VI, raising the possibility that synaptic protein loss is tightly correlated with tau burden. This is supported by studies in tau transgenic mice (Yoshiyama *et al*., 2007) and in human cohorts where synaptic protein loss only occurs at the highest Braak stages (Mukaetova-Ladinska *et al*., 2000). On the other hand, as the synaptophysin RIR, Braak stage, hippocampal sclerosis and the TDP-43 pathology measures all remained significantly associated with MMSE scores in our multiple logistic regression analysis, perhaps something more than pathological Alzheimer's disease is taking place and that synaptic protein loss in the perforant pathway and hippocampal sclerosis are independent factors that contribute to dementia in the oldest-old.

If cognitively normal individuals have age-related tangles and plaques, and these pathologies increase along with synaptic loss in dementia, what changes occur during CIND? CIND in the oldest-old may be associated with a greater substantial distribution of plaques as measured by Thal phase (Fig. 3A). Cognitively normal nonagenarians at Thal phase 2 have plaque deposition that has spread into layers 2/3 of the entorhinal cortex (Thal *et al*., 2006), suggesting that plaques are already present in the perforant pathway. In the CIND group, the median Thal phase was 3 indicating a greater plaque burden affecting the perforant pathway. As there is no concurrent increase in the Braak stage (median III), this may signify that plaques are having an earlier effect than tangles, which is consistent with numerous biomarker studies that show amyloid-β becomes abnormal before tau in cognitively normal individuals (Aisen, 2010; Jack *et al*., 2013).

CIND may also be associated with a decrease in glutamatergic synapses in the perforant pathway as measured by the VGLUT1 RIR (Fig. 3B). Although the VGLUT1 RIR can clearly distinguish the normal and dementia groups ($P = 0.034$), this measure does not distinguish the CIND from the dementia groups ($P = 0.747$), whereas the synaptophysin ($P < 0.001$) and SV2 RIRs ($P = 0.008$) still do (Table 2). The most parsimonious explanation is that non-glutamatergic neurons remain relatively unaffected during CIND whereas glutamatergic neurons experience synaptic loss. If true, this is consistent with previous work showing that observed presynaptic proteins, such as synaptophysin, remain constant or increase in earlier phases of illness before decreasing with dementia onset (Honer, 2003; Head *et al*., 2009). That the decrease in the VGLUT1 RIR is coincident with the increase in Thal phase may imply that plaques damage glutermatergic synapses. Of course, there are many possibilities including that the reduction in VGLUT1 may simply signify a loss of transporters, rather than the loss of glutamatergic afferents (Tannenberg *et al*., 2004).

Importantly, our study suggests that individuals with CIND are for the most part pathologically and synaptically indistinguishable from individuals with normal cognition. This being the case, pharmaceutical interventions that target tau and amyloid-β or protect synapses are all viable therapeutic strategies for a population at risk of dementia due to Alzheimer's disease. In addition, interventions designed to maintain a healthy synaptic system and a strict control of vascular risk factors may be good strategies to preserve cognitive function in nonagenarians and centenarians.

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