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RESEARCH ARTICLE

Quantitation and Mapping of Cerebral Detergent-Insoluble Proteins in the Elderly

Randall L. Woltjer[1]; Joshua A. Sonnen[2]; Izabela Sokal[2]; Lisa G. Rung[2]; Wan Yang[2]; John D. Kjerulf[2]; Danielle Klingert[2]; Charles Johnson[2]; Isaac Rhew[3]; Debbie Tsuang[3,4]; Paul K. Crane[5]; Eric B. Larson[5,6]; Thomas J. Montine[2,7]

[1] Department of Pathology and [7] Department of Neurology, Oregon Health Sciences University, Portland, Or.
[2] Department of Pathology, [4] Department of Psychiatry and Behavioral Sciences, and [5] Department of Medicine, University of Washington, Seattle, Wa.
[3] Puget Sound VAHMC, Veterans Affairs Northwest Network Mental Illness, Seattle, Wa.
[6] Group Health Cooperative, Center for Health Studies, Seattle, Wa.

Corresponding author:
Randall L. Woltjer, Department of Pathology, Oregon Health and Sciences University, 3181 SW Sam Jackson Park Road, Mail Code L113 Portland, OR 97239-3098 (E-mail: *woltjerr@ohsu.edu*)

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Abstract

Accumulation of abnormal protein aggregates, detergent-insoluble (DI) proteins and amyloid in the brain are shared features of many neurodegenerative diseases. Previous studies correlating DI proteins and cognitive performance are limited. We addressed these limitations using two sets of autopsy brains, one selected from our Alzheimer's Disease Research Center and the other an unselected series from Adult Changes in Thought (ACT), a population-based study of brain aging. We observed concentrations of 11 proteins and 6 protein variants that can be grouped into three highly correlated clusters: amyloid (A)β, tau and alpha-synuclein (α-syn). While abnormal proteins from each cluster independently correlated with cognitive performance in ACT participants, only increased soluble Aβ oligomers in temporal cortex and increased DI Aβ 42 and DI α-syn in prefrontal cortex were negatively correlated with cognitive performance. These data underscore the therapeutic imperative to suppress processes leading to accumulation of soluble Aβ oligomers, DI Aβ 42 and DI α-syn, highlight an at least partially independent contribution to cognitive impairment and raise the possibility that the priority for therapeutic targets may vary by brain region in a typical elderly US population.

INTRODUCTION

Accumulation of abnormal protein aggregates is a shared feature of many neurodegenerative diseases including common illnesses such as Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and Parkinson's disease (PD). Indeed, it is just this feature that has led several to propose that these common neurodegenerative diseases are "protein misfolding diseases" [reviewed in Agorogiannis *et al*. (1) and Soto and Estrada (27)]. These abnormal proteins are thought to share the feature of enhanced hydrophobicity and can form several abnormal assemblies including soluble aggregates, forms resistant to extraction with detergents and microscopically observable inclusions or amyloid deposits. The development of these protein abnormalities can be mechanistically related *in vitro*; however, the mechanistic connections *in vivo* are less clear but may be influenced by oxidative stress (23). Recently, we have found that the repertoire of detergent-insoluble (DI) proteins in late-onset Alzheimer's disease (LOAD) and early-onset autosomal dominant AD from mutations in presenilin 1 and 2 (*PS1* and *PS2*) consists of about 125 proteins, substantially larger than previously conceived (33). This suggests that the pathological processes that lead to these abnormal proteins may extend far beyond those that form inclusions or amyloids. One possibility is that while many proteins are altered to DI forms, only a small group of DI proteins has the necessary intrinsic properties or local environment necessary for progression to inclusions or amyloids (31, 34, 35, 37, 38). Thus, while only a few proteins may be capable of progressing from soluble aggregates to DI forms to amyloid, the number of proteins that can participate in some proximal aspects of this pathological biochemistry *in vivo* may be much larger.

Much debate has surrounded the relative functional significance of soluble aggregates, DI forms and inclusions to neurodegeneration, especially for Aβ peptides in AD (25). While this is not resolved, current evidence supports more prominent roles for soluble aggregates and DI forms in neuron dysfunction and death (3, 7, 13, 15, 30) and a less prominent, if not protective role, for inclusion formation [reviewed in Stefani and Dobson (28)]. Thus, it is unclear how to interpret the many studies that have associated histological or immunohistochemical data from these inclusions with clinical diagnosis or some rating of cognitive impairment. A few studies have attempted to correlate quantitative biochemical data on soluble Aβ oligomers (5, 12) or DI forms of Aβ species, tau

or alpha-synuclein (α-syn) with clinical diagnosis or a ranking of cognitive impairment (4). There are some limitations to these biochemical studies. First, none have attempted a comprehensive evaluation of soluble Aβ oligomers, DI Aβ species, tau and α-syn; moreover, none have considered other DI proteins that accumulate in AD brain (33). Second, none have attempted to put the results from these restricted analyses of DI proteins into context with other processes like vascular brain injury (VBI), which is a strong independent predictor of dementia in the elderly (26). Third, relatively few brains from individuals without dementia (<26 individuals) have been investigated in each of these studies. Fourth, associations have been drawn between biochemical data and clinical diagnosis or rating of dementia severity and, as far as we are aware, never to a continuous measure of cognitive performance. Finally, while some have evaluated brains from excellent clinicopathologic studies like the Religious Orders Study (4, 18), these samples may not be representative of the general population (36). We think this last point is especially important because the success or failure of future therapies will be judged on their effectiveness in the general population.

In this study, we attempted to address each of these points by quantifying a broad range of DI proteins in multiple brain regions from 54 individuals (35 not demented) who participated in a Seattle metropolitan area population-based study of brain aging and incident dementia, the Adult Changes in Thought (ACT) study, which provided biennial neuropsychological testing.

METHODS

Samples and case selection

The first set of participants was individuals who were seen as part of the Clinical Core of the Alzheimer's Disease Research Center (ADRC) at the University of Washington (UW). Assessment of mild cognitive impairment (MCI) exactly followed published criteria (21). Diagnosis of AD was made by consensus following established methods (16). The second set of participants was derived from the ACT study, which is a large population-based study of brain aging and incident dementia conducted in Group Health Cooperative, a well-established HMO in the Seattle metropolitan area. In this cohort, cognitively normal volunteers are enrolled at 65 years or older and are followed every 2 years with medical examination and cognitive screen with the Cognitive Assessment Screening Instrument (CASI) until diagnosis of dementia according to Diagnostic and Statistical Manual of Mental Disorders, revision IV (DSM-IV) criteria (14). These two studies have been approved by the institutional review boards at UW and the HMO.

Two sets of human post-mortem brain tissue collected in the Neuropathology Core of the UW ADRC were used: first, those from the Clinical Core of the UW ADRC and subsequently, those from the ACT study. First, we performed an extensive regional exploration of changes in protein level using serial extractions performed on a small number of selected cases from the Clinical Core of the UW ADRC. These cases were selected for study based on clinical diagnosis of control, MCI or LOAD, and the lack of Lewy body disease (LBD) or VBI. Characteristics of people whose tissue was selected for use in this study are presented in Table 1.

We used our initial analyses of results from the ADRC selected samples to identify specific regions in which differences were most prominent. We then sampled these regions from all consecutive autopsy cases from the ACT study that had frozen tissue through August 2006. Characteristics of ACT participants whose tissue was used are presented in Table 2. All autopsies were performed following appropriate informed consent and tissue used in accord with institutional review board-approved protocols.

In all cases, fresh frozen tissue was weighed and homogenized in ice-cold Tris/sucrose-containing buffer and was extracted as previously described (33). Fractions prepared were soluble in buffer, soluble in buffer +1% triton, insoluble in buffer +1% triton or were triton insoluble (TI) and insoluble in buffer +1% N-lauroylsarcosine or sarkosyl insoluble (SI).

Neuropathologic evaluation

Neuropathologic assessment of AD changes was performed according to National Institute on Aging-Reagan Institute consensus criteria (16, 21). We excluded cases with either (i) LBD as assessed by α-syn immunohistochemistry of brainstem (including substantia nigra), amygdala and middle frontal gyrus (MFG) (14 cases); or (ii) VBI as assessed by any grossly observed arterial territorial infarct, any grossly observed lacunar infarct or any microvascular infarct (MVI) observed according to our published scoring system (25 cases).

Protein quantification by Enzyme-Linked Immunosorbant Assay

All anti-β-amyloid antibodies used [anti-residues 17–24 (4G8), anti-β-amyloid 1-40 (Aβ40) (11A50-B10), and anti-β-amyloid 1-42 (Aβ42) (12F4)] were from Covance (Princeton, NJ, USA).

Table 1. Demographic, clinical and neuropathologic characteristics of selected Alzheimer's Disease Research Center (ADRC) autopsies. Abbreviations: LOAD = late-onset Alzheimer's disease; MCI = mild cognitive impairment; PMI = post-mortem interval; NP = neuritic plaque; CERAD = Consortium to Establish a Registry for Alzheimer's Disease.

Group	n	M:F	Age (year)	PMI (h)	Brain wt (g)	CERAD NP score*	Braak stage**
Control	5	2:3	88 ± 6	4.7 ± 0.8	1184 ± 187	Sparse (none–sparse)	II (II–IV)
MCI	5	2:3	89 ± 9	5.8 ± 1.1	1062 ± 136	Sparse (sparse–moderate)	IV (III–IV)
LOAD	4	2:2	81 ± 4	5.1 ± 0.4	1000 ± 82	Frequent (moderate–frequent)	VI (VI)

Data are mean ± standard deviation (SD) for age, PMI and brain weight (wt); patients were selected from the Alzheimer's Disease Research Center (ADRC) cohort. CERAD NP score and Braak stage are presented as mode (range). One-way analysis of variance (ANOVA) for age, PMI and brain weight all had $P > 0.05$. χ^2 test among the three groups for CERAD NP score had $^*P < 0.05$ and for Braak stage had $^{**}P < 0.0005$. None of these cases had Lewy body disease (LBD) or vascular brain injury (VBI).

Table 2. Clinical characteristics and pathological data from consecutive Adult Changes in Thought study autopsies through August 2006. Abbreviations: NS = not significant; CASI = Cognitive Assessment Screening Instrument; PMI = post-mortem interval; NP = neuritic plaque; MVI = microvascular infarct.

		Not demented	Demented	*P*-value
Total*		35	19	—
Age (year)†		87 ± 6	89 ± 5	NS‡
Male* (%)		16 (46%)	7 (37%)	NS§
Last CASI†		88 ± 7	61 ± 16	<0.0001‡
Last CASI to death (days)†		359 ± 162	431 ± 193	NS‡
PMI (h)†		4.9 ± 0.9	5.2 ± 1.1	
Brain weight (g)†		1172 ± 154	1097 ± 112	NS‡
CERAD NP score*	None	13 (37%)	2 (11%)	0.04¶
	Sparse	17 (49%)	10 (53%)	
	Moderate	5 (14%)	4 (21%)	
	Frequent	0 (0%)	3 (16%)	
Braak score (stage)*	0–II (A)	19 (54%)	5 (26%)	0.003¶
	III–IV (B)	15 (42%)	7 (37%)	
	V–VI (C)	1 (3%)	7 (37%)	
Amyloid angiopathy*	None	32 (19%)	11 (58%)	0.009¶
	1	1 (3%)	4 (21%)	
	2–3	2 (6%)	4 (21%)	
Isocortical Lewy body*	None	34 (97%)	15 (79%)	0.05¶
	Any	1 (3%)	4 (21%)	
Gross infarcts*	0	29 (83%)	12 (63%)	NS¶
	1	4 (11%)	5 (26%)	
	2	2 (6%)	0 (0%)	
	3	0 (0%)	2 (11%)	
MVI*	None	30 (86%)	9 (47%)	0.002¶
	1–2	5 (14%)	6 (32%)	
	3 or more	0 (0%)	4 (21%)	

NS has $P > 0.05$.
*Number.
†Mean ± standard deviation (SD).
‡t-Test.
§χ^2 test.
¶Fisher's exact test.

Anti-apolipoprotein E, anti-ubiquitin and anti-tau polyclonal antibodies were from Dako Corporation (Carpinteria, CA, USA). Anti-p181-phosphotau and anti-p212-phosphotau antibodies were from Pierce (Rockford, IL, USA), and anti-p231-phosphotau and anti-p396-phosphotau were from Biosource International (Camarillo, CA, USA). Anti-α-syn was from Labvision (Fremont, CA, USA). Anti-glial fibrillary acidic protein was from Novus Biologicals (Littleton, CO, USA). Anti-N^{ε}-(carboxymethyl)lysine (CML) and anti-malondialdehyde (MDA) were from Academy Biomedical (Houston, TX, USA). Anti-heat shock protein 70 (HSP70) was from Sigma (St. Louis, MO, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Chemicon (Temecula, CA, USA). Anti-14-3-3 and horseradish peroxidase-labeled secondary anti-goat antibody were from Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA). Horseradish peroxidase-labeled secondary anti-rabbit and anti-mouse were obtained from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were purchased from Sigma.

We selected to study 17 proteins or protein variants that have been previously described in the DI fraction from patients with AD based on commercial availability of specific antibodies that could be adapted to antibody capture assays. Antibody specificity was confirmed by Western blot of brain extracts. Antibody capture assays were carried out as described (6). Formic acid extracts of DI proteins were dried by vacuum centrifugation and were resolubilized by sonication in 20 vol of 5 M guanidine HCl, 100 mM Tris, pH 7.4, with 0.002% bromphenol blue added to confirm elimination of formic acid. These were further diluted 64-fold in 100 mM Tris, pH 7.4 with 0.05% azide and 0.002% bromphenol blue, and 100 μL was spotted onto 96-well plates and then incubated overnight at room temperature in a humidified chamber. Subsequently, the plates were washed twice with phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin (BSA) in PBS with 0.05% sodium azide and washed again with PBS. All antibodies were diluted 2000 times from the initial stock concentration before use in the capture assay. After washing three times, assays were developed using tetramethylbenzidine. Absorbances at 450 nm were determined using a Spectramax 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and results were normalized to a set of standard extracts from previously characterized brain tissue.

Quantification of soluble Aβ oligomers

Luminex assays for soluble Aβ oligomers were performed in S1 and S2 using reagents from Biosource International (catalog entry NON0679) exactly according to the manufacturer's instructions as we have previously described (35). This assay detects oligomeric protein with antibody A11 coupled with antibody 7N22 that binds

to an epitope contained within the amino acids 1–20 of Aβ peptides. Standards were provided by the manufacturer and standard curves were generated over 30–2000 pg/mL Aβ42 oligomers with a detection limit of 26 pg/mL.

Statistics

Statistical analyses were performed using Stata 9.0 (StataCorp LP, 4905 Lakeway Drive, College Station, TX, USA). Comparisons between groups were based on analysis of variance (ANOVA) for continuous variables and the chi-squared or Fisher's exact test for categorical variables. One-way ANOVA post hoc tests were performed using the Bonferroni correction. Soluble Aβ oligomers were assessed using robust regression. Candidate protein levels were divided into quartiles and were modeled as indicator variables using the lowest quartile as the reference group. Quartiles were used because we also tested for a trend in the association by modeling the quartiles of protein levels as a grouped linear variable. Robust regression by quartiles was also used to examine the association between candidate proteins and last CASI score. Stepwise robust regression was used to compute final models of the last CASI score. When referring to levels of significance, modest associations are defined as $0.05 > P > 0.01$ and strong associations are defined as $P < 0.01$.

RESULTS

ADRC cases

Our initial analysis utilized patients' tissue selected from the Clinical Core of the UW ADRC in an attempt to comprehensively replicate results from other previous studies of AD, validate the performance of our assays and expand our observations to multiple brain regions. Table 1 presents the characteristics of individuals whose tissue was used in this study. The pathological data are typical of those observed by us and others for cases from tertiary referral centers (2, 9, 19). We used Braak stage as the primary means of stratification: three stage A (transentorhinal) individuals who were all clinically diagnosed as controls, seven stage B (hippocampal) individuals who were clinically diagnosed as MCI (n = 5) or controls, and four stage C (isocortical) patients who were all clinically diagnosed with probable AD and who also met NIA-Reagan neuropathologic criteria for high likelihood of AD (22). We first determined the anatomical distribution of soluble Aβ oligomers (Figure 1). Soluble Aβ oligomer concentration varied over orders of magnitude with respect to brain region and Braak stage, and increased in concentration in association with increasing Braak stage most closely in the MFG and in the superior and middle temporal gyri (SMTG) ($P < 0.01$), modestly related to Braak stage in inferior parietal lobule (IPL) ($P < 0.05$), and not related to Braak stage in occipital cortex (OC), caudate (Cd) or cerebellum (Cb).

Our next series of analyses focused on DI forms of proteins from the UW ADRC cases. Our experimental approach was to extract tissue as described above, to solubilize remaining protein with 70% formic acid (FA) and then to quantify the relative amount of protein in the FA extract by ELISA. We used ELISAs to quantify 17 different proteins or protein modifications (listed in Table 3). It is important to emphasize that none of the ADRC cases had LBD or VBI. Five proteins had DI forms detected in all four cortical regions of

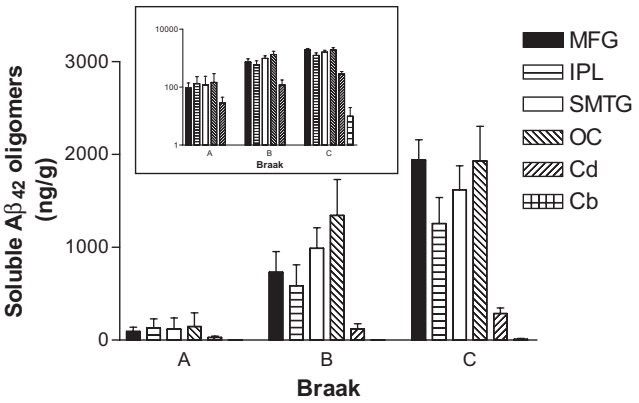

Figure 1. Data are average ± standard error of the mean (SEM) concentrations (ng/g) of soluble amyloid (A)β oligomers plotted on linear scale (log scale of the same data in inset) and stratified by Braak stage in six regions of the brain from 14 individuals who died with no cognitive impairment, with mild cognitive impairment (MCI) or with late-onset Alzheimer's disease (LOAD) (n = 3 for Braak A, n = 7 for Braak B and n = 4 for Braak C); patients were selected from the Alzheimer's Disease Research Center (ADRC) cohort. Two-way analysis of variance (ANOVA) had $P < 0.0001$ for Braak stage and brain region as $P < 0.05$ for interaction between these two terms. We pursued post hoc one-way ANOVA for each region across Braak stages. In these analyses, middle frontal gyrus (MFG) and superior and middle temporal gyri (SMTG) cortex yielded similar results of $P < 0.01$ for soluble Aβ oligomers across the three Braak stages; Bonferroni-corrected repeat paired comparisons had $P < 0.01$ for Braak stage A vs. Braak stage C for both regions of the brain, but Braak stage B vs. Braak stage C only for MFG. One-way ANOVA had $P < 0.05$ for inferior parietal lobule (IPL), but no significant corrected repeat paired comparisons, while one-way ANOVA for occipital cortex (OC), caudate (Cd) and cerebellum (Cb) had $P > 0.05$.

Braak stage C AD patients (Figure 2): Aβ40, Aβ42, tau, ubiquitin and apoE. All five DI proteins were strongly associated with Braak stage ($P < 0.0001$). DI protein levels for ubiquitin and apoE varied modestly across brain region ($P < 0.05$). Across brain regions, Aβ40 levels did not differ; DI ubiquitin and apoE differed modestly ($P < 0.05$), and DI Aβ42 and tau levels varied strongly ($P < 0.0001$). Corrected post tests among the four cerebral cortical regions showed that DI Aβ40 and Aβ42 increased successively from Braak stage A to B and from Braak stage B to C ($P < 0.05$ for each), while DI tau, ubiquitin and apoE did not increase significantly from Braak stage A to B (with the exception of occipital DI ubiquitin), but did increase from Braak stage B to C ($P < 0.01$ for each). These findings suggest that the four regions of cerebral cortex had similar distributions of all five DI proteins with respect to Braak stage, but the Cd and Cb differed from the cerebral cortex and from each other. Moreover, these ELISA results, which confirmed our previous finding from Western blots on extracts from the temporal lobe (33), demonstrate that cerebral cortical DI Aβ species increase significantly from Braak stage A to B, while cerebral cortical DI tau increases at late stages of AD progression.

In summary, our results from relatively "pure" AD cases obtained through a tertiary referral center closely agreed with those previously published by others (4, 18). Concentrations of soluble Aβ oligomers paralleled the extent of tissue involvement by AD;

Table 3. Robust regressions for quartiles of protein levels in middle frontal gyrus (MFG) and superior and middle temporal gyri (SMTG) from consecutive Adult Changes in Thought study autopsies through August 2006. Abbreviations: NA = not applicable; NS = not significant; A= amyloid; TI= triton insoluble; SI = sarkosyl insoluble; CML = N^{ε}-(carboxymethyl)lysine.

			Soluble Aβ oligomers	TI tau	TI α-syn
Protein	Region	Fxn			
Aβ (4G8)	MFG	TI	++++*	NS	NS
		SI	++++**	NS	NS
	SMTG	TI	++++**	++++**	+
		SI	++++**	++++**	+
Aβ42	MFG	TI	++++**	NS	NS
		SI	++++**	NS	NS
	SMTG	TI	++++**	++++**	NS
		SI	++++**	++++**	++
Aβ40	MFG	TI	++++ *	NS	NS
		SI	++++	NS	NS
	SMTG	TI	+*	NS	NS
		SI	NS	NS	NS
apoE	MFG	TI	+++*	NS	NS
		SI	++*	NS	NS
	SMTG	TI	+++**	++*	NS
		SI	NS	NS	NS
Tau	MFG	TI	NS	NA	NS
		SI	+	NA	NS
	SMTG	TI	NS	NA	+
		SI	NS	NA	NS
Tau-P181	MFG	TI	NS	+*	++
		SI	NS	NS	NS
	SMTG	TI	+*	+	++++**
		SI	NS	NS	NS
Tau-P212	MFG	TI	NS	NS	NS
		SI	NS	+*	NS
	SMTG	TI	++++**	++**	++++**
		SI	NS	+	NS
Tau-P231	MFG	TI	NS	++	NS
		SI	NS	+ *	NS
	SMTG	TI	+*	++++**	++++**
		SI	NS	++++**	NS
Tau-P396	MFG	TI	+*	NS	NS
		SI	+*	+*	NS
	SMTG	TI	+	++++**	++
		SI	NS	++++**	NS
Ubiquitin	MFG	TI	NS	NS	NS
		SI	NS	NS	NS
	SMTG	TI	NS	NS	++*
		SI	NS	NS	NS
α-Syn	MFG	TI	NS	NS	NA
	SMTG	TI	+	++*	NA
14-3-3	MFG	TI	NS	NS	++++**
	SMTG	TI	NS	NS	++++**
HSP70	MFG	TI	NS	NS	++++**
	SMTG	TI	++**	++	++++**
CML	MFG	TI	NS	NS	++++*
	SMTG	TI	NS	NS	++++**
GAPDH	MFG	TI	NS	NS	++*
	SMTG	TI	NS	+	NS
MDA	MFG	TI	NS	NS	+++*
	SMTG	TI	NS	NS	+++**
GFAP	MFG	TI	NS	NS	NS
	SMTG	TI	NS	+	NS

Results are from robust regressions for quartiles of 17 protein concentrations in TI or SI fractions (Fxn) from the MFG or SMTG cortex (rows) vs. quartiles for soluble Aβ oligomers, TI tau and TI alpha-synuclein (α-syn) in the corresponding brain region (columns). SI α-syn, SI 14-3-3, SI HSP70, SI CML, SI GAPDH, SI MDA and SI GFAP were all below the limit of detection (BLD) in MFG and SMTG, and so only TI data are presented for these proteins. *P*-values for interquartile comparison are represented with "–" for $P > 0.05$, "+" for $P < 0.05$, "++" for $P < 0.01$, "+++" for $P < 0.001$ and "++++" for $P < 0.0001$. *P*-value for trend across all quartiles: *<0.05 or **<0.01.

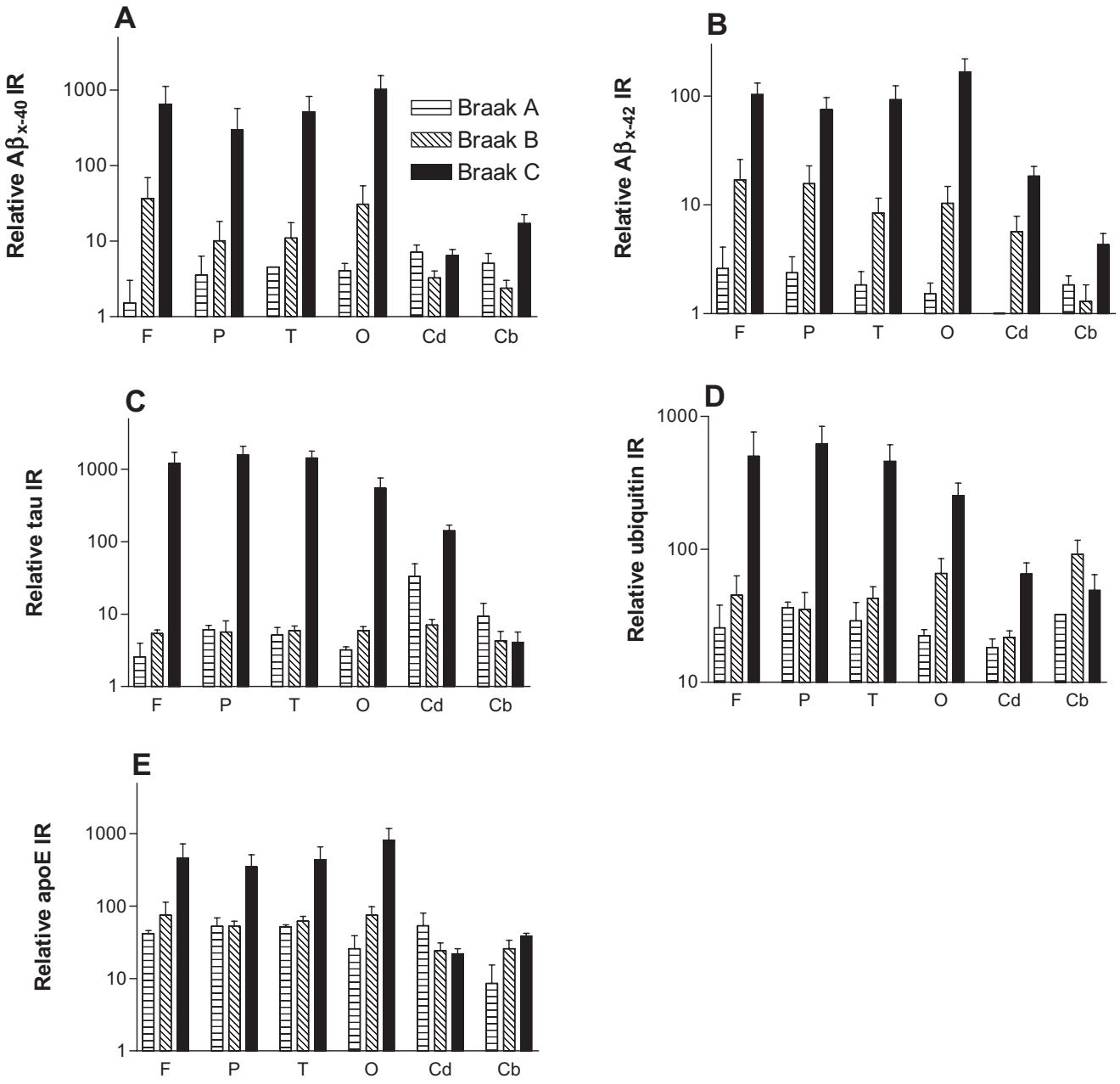

Figure 2. Data are average ± standard error of the mean (SEM) relative amounts of five proteins measured by ELISA immunoreactivity (IR) in the sarkosyl-insoluble (SI) fraction from six regions of the brain: middle frontal gyrus (MFG) (F), inferior parietal lobule (IPL) (P), superior and middle temporal gyri (SMTG) (T), occipital cortex (OC) (O), caudate (Cd) nucleus and cerebellum (Cb). Fourteen people were selected from the Alzheimer's Disease Research Center (ADRC) cohort who died without cognitive impairment, mild cognitive impairment (MCI) or late-onset Alzheimer's disease (LOAD). Note that data are plotted on a log scale. Two-way analysis of variance (ANOVA) for all five SI proteins had $P < 0.0001$ for Braak stage. SI Aβ40 was not significantly related to brain region ($P > 0.05$); SI ubiquitin and apoE were modestly related to brain region ($P < 0.05$ for each), and SI Aβ42 and tau were strongly associated with brain region ($P < 0.0001$ for each). Interaction between Braak stage and brain region also segregated into these same three groups: $P > 0.05$ for Aβ40, $P < 0.05$ for ubiquitin and apoE, and $P < 0.0001$ for Aβ42 and tau. One-way ANOVA post tests with Bonferroni-corrected repeat paired comparisons in each of the four cerebral cortical regions showed that SI Aβ40 and Aβ42 increased successively from Braak stage A to Braak stage B and from Braak stage B to Braak stage C ($P < 0.05$ for each repeat paired comparison). In contrast, SI tau, ubiquitin and apoE did not increase significantly from Braak stage A to Braak stage B (with the exception of occipital SI ubiquitin, $P < 0.01$), but did increase from Braak stage B to Braak stage C ($P < 0.01$ for each repeat paired comparison).

concentrations of DI Aβ species increased early in the course of AD, and concentrations of other DI proteins increased in later stages of AD. In addition, we extended our analysis to other regions of the brain not usually considered in the pathogenesis of AD (Cd and Cb) and showed that these regions also are characterized by DI protein accumulation, but at a lower level than cerebral regions.

ACT cases

Our next series of analyses applied this same analytic plan to all autopsies with frozen tissue obtained from a population-based study of brain aging and dementia in the metropolitan Seattle region through August 2006 (Table 2). As our anatomical map in the ADRC cases pointed to the MFG and SMTG as the most informative regions, we focused on these two regions of the brain in the ACT series. As expected, this is a more complex sample with a dominant presence of AD pathological changes, but is now accompanied by commonly comorbid conditions that also are correlated with dementia: VBI and LBD (26).

We first analyzed our data for interrelationships among DI proteins or soluble Aβ oligomers with the idea that unexpected associations may point us toward new mechanistic hypotheses (Table 3). Our analysis identified three clusters. We termed the first cluster the "Aβ cluster." Here we used soluble Aβ oligomers as the principal variable and observed moderate to strong correlations with DI Aβ species and DI Aβ42, as well as weak to moderate associations with Aβ40 and apoE. All of these proteins are associated with amyloid plaques and may be anticipated from published reports that describe other mechanistic and biochemical associations between Aβ and apoE [reviewed in Holtzman (8)]. Our second cluster was the "tau cluster," with TI tau as the principle variable. Here we observed correlation of DI tau with especially p231- and p396-phosphotau. These mid- to late-stage phosphorylated epitopes were present predominantly in the SMTG and may correlate with higher levels of pretangle and neurofibrillary tangle burdens. Our third cluster was called the "α-syn cluster" as it had TI α-syn as its principle variable. We identified associations between α-syn and early- to mid-stage phosphorylated epitopes of tau in the SMTG, but not in the MFG. In distinction to DI tau and Aβ species, we observed strong correlation of α-syn with 14-3-3 protein, HSP70 and CML immunoreactivity as well as weak correlation with GAPDH and MDA immunoreactivity in both SMTG and MFG, suggesting specific hydrophobic interactions among pathologic forms of α-syn and these other proteins or protein modification.

Next, we examined the possible association between abnormal protein levels from each of the above clusters in MFG or SMTG and last CASI score regardless of clinical diagnosis (Table 4). There are limitations to this type of analysis including the screening nature of CASI and the unknown effects of the interval between last evaluation and death. Despite these imperfections, this approach offers the opportunity to compare biochemical data with a systematic evaluation of cognitive function. Our results showed that concentration of pathologic Aβ species in the MFG and SMTG were inversely associated with the last CASI score. As expected, this relationship appeared stronger for pan-Aβ species and Aβ42 than Aβ40. Interestingly, these associations differed between cerebral regions. For example, soluble Aβ oligomers were more strongly associated with the last CASI score in the SMTG than in the MFG. In contrast, overall tau and P-taus were more strongly associated with last CASI score in the MFG than in the SMTG.

Given this apparent difference between cerebral regions, in our final analysis, we performed stepwise robust regression of our biochemical end points with the last CASI score for both the MFG and SMTG (Table 5). We observed that the optimal model for MFG included DI Aβ42 and DI α-syn, which were strongly associated with the last CASI. In contrast, the optimal model for SMTG included soluble Aβ oligomers, but none of the DI proteins.

Table 4. Robust regression of last Cognitive Assessment Screening Instrument (CASI) score by quartiles of protein concentration from consecutive Adult Changes in Thought study autopsies through August 2006. Abbreviations: A = amyloid; α-syn = alpha-synuclein; ND = not detected; NS = not significant; TI = triton insoluble; SI = sarkosyl insoluble; MFG = middle frontal gyrus; SMTG = superior and middle temporal gyri.

Protein	Fraction	MFG	SMTG
Aβ species	Soluble oligomers	+	+++**
	TI (4G8)	+++**	+**
	SI (4G8)	++++**	++*
Aβ42	TI	++++**	NS
	SI	NS	++*
Aβ40	TI	NS	NS
	SI	+	NS
Tau	TI	NS	NS
	SI	++++**	NS
Tau-P181	TI	NS	+*
	SI	+*	NS
Tau-P212	TI	++++*	NS
	SI	NS	NS
Tau-P231	TI	+*	NS
	SI	NS	NS
Tau-P396	TI	++++**	NS
	SI	NS	+
α-Syn	TI	++**	+*
	SI	ND	ND

Results are from robust regression of last CASI score vs. 10 TI or SI proteins in different fractions from the MFG and the SMTG. *P*-values for third quartile to fourth quartile are represented with "–" for $P > 0.05$, "+" for $P < 0.05$, "++" for $P < 0.01$, "+++" for $P < 0.001$ and "++++" for $P < 0.0001$. *P*-values for all other interquartile comparisons were NS. *P*-value for trend across all quartiles: *<0.05 or **<0.01.

Table 5. Optimal models for middle frontal gyrus (MFG) or superior and middle temporal gyrus (SMTG) cortex using stepwise multivariate robust regression of last Cognitive Assessment Screening Instrument (CASI) score from consecutive Adult Changes in Thought study autopsies through August 2006. Abbreviations: A = amyloid; α-syn = alpha-synuclein; DI = detergent insoluble.

Region	Protein	*t* statistic	*P*-value
MFG	DI Aβ42	–4.2	<0.0001
	DI α-syn	–3.6	<0.001
SMTG	Soluble Aβ oligomers	–3.9	<0.0001

DISCUSSION

A dominant theory for the molecular pathogenesis of dementia in the elderly is that these diseases are characterized by the accumulation of protein aggregates [reviewed in Agorogiannis *et al* (1) and Soto and Estrada (27)]. While genetic associations from rare autosomal dominant forms of neurodegenerative diseases have highlighted a central role for abnormal forms of Aβ, tau and α-syn, proteomic studies have shown the participation of many more abnormally hydrophobic proteins, at least in AD. Moreover, to our knowledge, investigations of diseases characterized by these DI proteins have utilized tissue from patients referred to tertiary medical centers, individuals from a religiously oriented nursing home or from a Roman Catholic clergy (4, 18, 19). These limitations to generalizablility highlight potentially critical gaps in our knowledge as they undermine confidence in extrapolation of results that support the DI protein theory of neurodegeneration to the general population of elderly for whom prevention and treatment is ideally targeted (29).

In this study, we used two sets of autopsy brains to compare directly a large subset of the DI proteins that characteristically accumulate in common dementing illnesses and to apply this new knowledge to a population-based series. Our results confirmed and extended the findings of others when restricted to cases selected from our ADRC (4, 18, 19). Our initial experiments examined a large series of proteins commonly invoked in the AD literature in cases selected from our ADRC. Our principal goal here was to test the reproducibility of others' findings (4, 5, 18, 19) and in doing so, to validate our approach. Similar to others, we observed that soluble Aβ oligomers and DI forms of Aβ42, tau, ubiquitin and apoE all increased as AD progressed (4, 5, 18, 19), although abnormal Aβ species increased significantly earlier in the course of AD than did these other DI proteins, as we observed previously (33). We also observed that the processes of AD, as assessed by the accumulation of these abnormal proteins, involve Cd and Cb, although at lower levels than in the cerebral cortex. These results are consistent with biochemical and histological data from us and from others (32) showing that these regions are involved by AD. Overall, these data served to validate our methods.

We applied our validated methods to an unselected series of brain autopsies from ACT, a population-based study of brain aging and dementia. Here, we observed that the concentrations of 17 different proteins or protein variants can be grouped into three highly correlated clusters: Aβ, tau and α-syn. Moreover, while proteins from each of these clusters independently correlated with the last CASI score in our population-based series, only soluble Aβ oligomers in the SMTG and DI Aβ42 and α-syn in the MFG were significantly correlated with cognitive impairment when assessed by multivariate analysis. These data underscore the therapeutic imperative to suppress processes leading to the accumulation of soluble Aβ oligomers as well as DI Aβ42 and DI α-syn.

Soluble oligomeric species of Aβ are now considered likely candidates for early changes of AD (5, 12, 20). Others have quantified relative differences in soluble Aβ42 oligomers using dot blot immunoassays that detect AD diffusible ligands (ADDLs) in the frontal cortex from five individuals with AD and in five controls (5). These investigators showed an average 12-fold greater ADDL concentration in AD patients and a maximum spread between individuals from these two groups of approximately 70-fold. In a subsequent series of experiments, this same group used the same approach and observed an average 11-fold increase in the frontal cortex that was statistically significant and an average 2.7-fold nonsignificant increase in the Cb between AD patients and controls (12). Our completely different assay that uses internal standards and is commercially available is in good agreement with these results. We observed in the frontal cortex an average of 93 ng/g soluble Aβ oligomers in Braak stage A individuals and 1941 ng/g soluble Aβ oligomers in Braak stage C individuals; this 21-fold difference was statistically significant. Our results in the Cb were somewhat different from these other investigators, but still are in broad agreement. As we used an authentic standard, we were able to set a lower limit of detection for our assay. Cb in both Braak stage A and B groups had soluble Aβ oligomers below the limit of detection (BLD). However, soluble Aβ oligomers were detectable in the Cb of patients with AD (Braak stage C). In our work, we have extended the anatomic map of soluble Aβ oligomers to four other brain regions and have shown that concentrations of this Aβ species are highly related to brain region. In addition, we have, for the first time we are aware, quantified brain levels of soluble Aβ oligomers in an intermediate stage of AD pathologic change and have shown that the concentration of these pathogenically important aggregates was highly associated with Braak stage. The latter is an important discovery because it firmly associates extensive accumulation of soluble Aβ oligomers throughout the cerebral cortex in individuals with early pathologic changes of AD without clinical dementia.

Our next step was to investigate a large series of consecutive autopsies from a population-based study of brain aging and incident dementia in the Seattle area. These cases provide a unique opportunity to evaluate the potential functional significance of AD- and LBD-associated DI proteins in typical urban and suburban US populations. Our results show that the concentrations of 11 proteins and 6 protein variants correlated with each other within three clusters: Aβ, tau and α-syn clusters. Our results from the Aβ cluster serve to further validate our approach because we identified correlated proteins already known to interact mechanistically, that is, Aβ species and apoE. The α-syn cluster was somewhat unexpected, although some biochemical and immunohistochemical data have shown interaction among HSP70 or 14-3-3 with α-syn in LBD (10, 11, 24). The very strong association between DI α-syn and CML was surprising and raises the possibility that advanced glycation end products may contribute to the generation of DI α-syn (17, 33). There were only weak associations among members of the three clusters and so these data did not illuminate possible shared processes that lead to formation of DI Aβ, tau and α-syn.

While these data showed that accumulation of these abnormal proteins in the brain is characteristic of commonly occurring neurodegenerative disease in the general population of elderly individuals, our ultimate goal was to estimate the relative functional significance of these DI protein-related diseases. We performed univariate robust regression analyses to assess the association between last CASI score and 10 abnormal proteins (soluble Aβ oligomers and 9 DI proteins) in MFG or SMTG. We observed that soluble Aβ oligomers and each of the DI proteins in either MFG or SMTG correlated to varying degrees of significance with last CASI score. Our final analysis sought to determine the relative significance of these several significant univaritate correla-

tions to the last CASI score by entering them into a stepwise multivariate logistical regression model. In this model, the abnormal proteins that remained independently associated with the last CASI were reduced to DI Aβ42 and DI α-syn in MFG and soluble Aβ oligomers in SMTG. None of the other DI proteins, including tau, were associated with cognitive performance in our multivariate analysis, perhaps a reflection of their appearance later in the course of AD and possibly derivative role in these most common forms of cognitive impairment in the elderly. While DI tau concentration in these cerebral cortical regions was not associated with cognitive performance, it is important to note that the Braak stage for neurofibrillary tangles (NFTs) is a strong correlate of dementia in this group (26), perhaps highlighting significant differences between DI and NFT tau as well as the importance of anatomical distribution outside of these cerebral cortical regions. These data suggest an unexpected regional significance of abnormal Aβ and α-syn accumulation between frontal and temporal cortex, raising the possibility that these abnormal proteins have varying pathophysiological significance in these different regions of the cerebrum.

There are limitations to our study. One concern could be that our autopsy cohort does not reflect the ACT cohort. However, we have already shown that other than being older and therefore more likely to be demented, the autopsy cohort analyzed here does not differ significantly from the ACT cohort with respect to gender, education, apoE allele or marital status (26). Another limitation of our study is the use of CASI to screen for cognitive abilities. Like any large population-based study, ACT has struck a balance between quantity and measurement precision, that is, the size of the cohort and the need to avoid excessive subject burden which could lead to dropout, and a less representative sample practically limits the amount of cognitive testing that can be performed. So, while there are limitations to the CASI, it is nevertheless a widely used instrument to gauge cognitive function in large populations that is systematically applied to members of ACT.

Despite these limitations, our approach provides a unique perspective on the molecular pathogenesis of cognitive impairment and dementia in a typical population of US urban and suburban elderly individuals. The most parsimonious interpretation of our data is that the molecular targets for preventing and treating cognitive impairment and dementia in the general population of elderly individuals include those processes that lead to soluble Aβ oligomer formation, DI Aβ42 accumulation and DI α-syn accumulation.

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REFERENCES

1. Agorogiannis EI, Agorogiannis GI, Papadimitriou A, Hadjigeorgiou GM (2004) Protein misfolding in neurodegenerative diseases. *Neuropathol Appl Neurobiol* **30**:215–224.
2. Berg L, McKeel DW Jr, Miller JP, Storandt M, Rubin EH, Morris JC *et al* (1998) Clinicopathologic studies in cognitively healthy aging and Alzheimer's disease: relation of histologic markers to dementia severity, age, sex, and apolipoprotein E genotype. *Arch Neurol* **55**:326–335.
3. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem* **277**:32046–32053.
4. Forman MS, Mufson EJ, Leurgans S, Pratico D, Joyce S, Leight S *et al* (2007) Cortical biochemistry in MCI and Alzheimer disease: lack of correlation with clinical diagnosis. *Neurology* **68**:757–763.
5. Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE *et al* (2003) Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc Natl Acad Sci USA* **100**:10417–10422.
6. Harlow E, Lane D (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Woodbury.
7. Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM *et al* (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci* **19**:8876–8884.
8. Holtzman DM (2004) In vivo effects of ApoE and clusterin on amyloid-beta metabolism and neuropathology. *J Mol Neurosci* **23**:247–254.
9. Honig LS, Kukull W, Mayeux R (2005) Atherosclerosis and AD: analysis of data from the US National Alzheimer's Coordinating Center. *Neurology* **64**:494–500.
10. Kawamoto Y, Akiguchi I, Nakamura S, Honjyo Y, Shibasaki H, Budka H (2002) 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. *J Neuropathol Exp Neurol* **61**:245–253.
11. Klucken J, Shin Y, Masliah E, Hyman BT, McLean PJ (2004) Hsp70 reduces alpha-synuclein aggregation and toxicity. *J Biol Chem* **279**:25497–25502.
12. Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL *et al* (2004) Synaptic targeting by Alzheimer's-related amyloid beta oligomers. *J Neurosci* **24**:10191–10200.
13. Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M *et al* (1998) Diffusible, nonfibrillar ligands derived from abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* **95**:6448–6453.
14. Larson EB, Wang L, Bowen JD, McCormick WC, Teri L, Crane P, Kukull W (2006) Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older. *Ann Intern Med* **144**:73–81.
15. Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A *et al* (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* **440**:352–357.
16. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**:939–944.
17. Munch G, Luth HJ, Wong A, Arendt T, Hirsch E, Ravid R, Riederer P (2000) Crosslinking of alpha-synuclein by advanced glycation endproducts–an early pathophysiological step in Lewy body formation? *J Chem Neuroanat* **20**:253–257.
18. Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *Jama* **283**:1571–1577.
19. Nelson PT, Jicha GA, Schmitt FA, Liu H, Davis DG, Mendiondo MS *et al* (2007) Clinicopathologic correlations in a large Alzheimer

disease center autopsy cohort: neuritic plaques and neurofibrillary tangles "do count" when staging disease severity. *J Neuropathol Exp Neurol* **66**:1136–1146.
20. Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM (2003) Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging* **24**:1063–1070.
21. Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E (1999) Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol* **56**:303–308.
22. Reagan R (1998) Ronald and Nancy Reagan Institute of the Alzheimer's Association and National Institute on Aging Working Group on biological markers of Alzheimer's disease. Consensus report on the Working Group on 'molecular and biochemical markers of Alzheimer's disease'. *Neurobiol Aging* **19**:109–116.
23. Roberti MJ, Bertoncini CW, Klement R, Jares-Erijman EA, Jovin TM (2007) Fluorescence imaging of amyloid formation in living cells by a functional, tetracysteine-tagged alpha-synuclein. *Nat Methods* **4**:345–351.
24. Shirakashi Y, Kawamoto Y, Tomimoto H, Takahashi R, Ihara M (2006) alpha-synuclein is colocalized with 14-3-3 and synphilin-1 in A53T transgenic mice. *Acta Neuropathol* **112**:681–689.
25. Skovronsky DM, Lee VM-Y, Trojanowski JQ (2006) Neurodegenerative diseases: new concepts of pathogenesis and their therapeutic implications. *Annu Rev Pathol: Mech Dis* **1**:151–170.
26. Sonnen JA, Larson EB, Crane PK, Haneuse S, Li G, Schellenberg GD *et al* (2007) Pathological correlates of dementia in a longitudinal, population-based sample of aging. *Ann Neurol* **62**:406–413.
27. Soto C, Estrada LD (2008) Protein misfolding and neurodegeneration. *Arch Neurol* **65**:184–189.
28. Stefani M, Dobson CM (2003) Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med* **81**:678–699.
29. Tsuang D, Simpson KL, Li G, Barnhart RL, Edland SD, Bowen J *et al* (2005) Evaluation of selection bias in an incident-based dementia autopsy case series. *Alzheimer Dis Assoc Disord* **19**:67–73.
30. White JA, Manelli AM, Holmberg KH, Van Eldik LJ, Ladu MJ (2005) Differential effects of oligomeric and fibrillar amyloid-beta 1-42 on astrocyte-mediated inflammation. *Neurobiol Dis* **18**:459–465.
31. Winton MJ, Joyce S, Zhukareva V, Practico D, Perl DP, Galasko D *et al* (2006) Characterization of tau pathologies in gray and white matter of Guam Parkinsonism-dementia complex. *Acta Neuropathol* **111**:401–412.
32. Wolf DS, Gearing M, Snowdon DA, Mori H, Markesbery WR, Mirra SS (1999) Progression of regional neuropathology in Alzheimer disease and normal elderly: findings from the Nun study. *Alzheimer Dis Assoc Disord* **13**:226–231.
33. Woltjer RL, Cimino PJ, Boutte AM, Schantz AM, Montine KS, Larson EB *et al* (2005) Proteomic determination of widespread detergent-insolubility including Abeta but not tau early in the pathogenesis of Alzheimer's disease. *FASEB J* **19**:1923–1925.
34. Yamazaki M, Hasegawa M, Mori O, Murayama S, Tsuchiya K, Ikeda K *et al* (2005) Tau-positive fine granules in the cerebral white matter: a novel finding among the tauopathies exclusive to Parkinsonism-dementia complex of Guam. *J Neuropathol Exp Neurol* **64**:839–846.
35. Yang W, Woltjer RL, Sokal I, Pan C, Wang Y, Brodey M *et al* (2007) Quantitative proteomics identifies surfactant-resistant alpha-synuclein in cerebral cortex of Parkinsonism-dementia complex of Guam but not Alzheimer's disease or progressive supranuclear palsy. *Am J Pathol* **171**:993–1002.
36. Zaccai J, Ince P, Brayne C (2006) Population-based neuropathological studies of dementia: design, methods and areas of investigation–a systematic review. *BMC Neurol* **6**:2.
37. Zhukareva V, Mann D, Pickering-Brown S, Uryu K, Shuck T, Shah K *et al* (2002) Sporadic Pick's disease: a tauopathy characterized by a spectrum of pathological tau isoforms in gray and white matter. *Ann Neurol* **51**:730–739.
38. Zhukareva V, Joyce S, Schuck T, Van Deerlin V, Hurtig H, Albin R *et al* (2006) Unexpected abundance of pathological tau in progressive supranuclear palsy white matter. *Ann Neurol* **60**:335–345.