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Frontal Cortex and Hippocampal γ-Secretase Activating Protein Levels in prodromal Alzheimer's disease

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Abstract

Background—Beta-amyloid (Aβ) is the product of concerted cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. However, the molecular mechanisms that regulate this process are not well understood. Recently evidence was reported that γ-secretase activating protein (GSAP, 16 kDa), derived of a larger precursor protein (98 kDa), plays a role in Aβ metabolism through a mechanism involving its interaction with both γ-secretase and APP. However, a detailed evaluation of GSAP protein levels and their association with clinical and neuropathological variables are lacking during clinical progression of Alzheimer's disease (AD).

Methods—We quantified levels of the GSAP precursor (98 kDa) and its active form (16 kDa) in frontal cortex and hippocampus, areas displaying extensive Aβ and neurofibrillary tangle pathology in subjects that came to autopsy with a premortem clinical diagnosis of non-cognitive impairment, mild cognitive impairment, mild to moderate AD and severe AD using western blotting.

Results—Analysis found that 98 kDa GSAP levels were increased, while 16 kDa were reduced in the frontal cortex of severe AD subjects. By contrast, GSAPs levels remained stable in the hippocampus. Frontal cortex and hippocampal GSAP 98 kDa and 16 kDa levels were not associated with Aβ, NFT and neuropathological criteria across clinical groups. Interestingly, only neocortical 98 kDa GSAP values showed a significant correlation with mini-mental state examination and episodic memory scores.

Conclusions—These data demonstrate that GSAP proteins are differentially dysregulated in sAD but only the full-length form was associated with cognitive tests in AD.

Keywords

Alzheimer's disease; γ-secretase; GSAP; frontal cortex; hippocampus; western-blot; mild-cognitive impairment

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Conflict of interest

The authors declare no conflicts of interest

INTRODUCTION

β-amyloid (Aβ) aggregates forming plaques follow the sequential cleavage of the amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase enzymes [1]. However, little is known about the mechanism involved in the γ-secretase substrate specificity and the regulation of the cleavage of the APP carboxy-terminal fragment (APP-CTF) to produce Aβ species. Several endogenous proteins have been reported to selectively modulate Aβ production [2–4], including the γ-secretase activating protein (GSAP) [5]. GSAP is synthetized from a larger 98 kDa protein, previously termed pion homologue protein (PION), which is rapidly processed into the predominant 16 kDa active form [5], resulting in the modulation of γ-secretase-Aβ production by its interaction with presenilin 1 (PS1) and APP-CTF without affecting notch cleavage [5]. *In vitro* and *in vivo* studies have demonstrated that changes in GSAP levels regulate Aβ production [5–7]. Studies in transgenic mouse models of AD have shown that pharmacological (using the kinase inhibitor imatinib) or genetic reduction of GSAP leads to a decrease in Aβ production, plaque development and tau phosphorylation [5–7]. GSAP immunoreactivity has been associated with neuronal PS1 and Aβ immunopositive plaques in the frontal cortex and hippocampus in AD [8]. Frontal cortex GSAP levels are significantly increased in patients with Down syndrome compared to age-match controls [9]. These findings suggest GSAP as a key enzyme in the synthesis of Aβ and unlike the pharmacological inhibition of γ-secretase, GSAP represents a more selective target for a safer anti-Aβ therapy. Despite its therapeutic potential, whether GSAP levels are altered early in the progression of AD remains unknown. In the present study, we examined protein levels of the full length or precursor (98 kDa) and the active form (16 kDa) of GSAP in frontal cortex and hippocampus, in subjects who died with a premortem diagnosis of non-cognitive impairment (NCI), mild-cognitive impaired (MCI), mild to moderate AD (mAD) and severe AD (sAD). These data were correlated with cognitive and neuropathological criteria.

METHODS

Subjects

Frontal cortex (Brodmann's area 10) and hippocampus were obtained from European American descendants who died with an ante-mortem clinical diagnosis of NCI (n=14), MCI (n=5 amnestic MCI and 11 non-amnestic MCI), and mAD (n=13) from the Rush Religious Orders Study (RROS). Additional cases with a clinical diagnosis of severe AD (sAD; n=13) were examined from the Rush Alzheimer's Disease Center (RADC). Human Research Committees of Rush University Medical Center approved this study.

Clinical and pathological analysis

Clinical evaluation included the mini-mental state examination (MMSE), episodic memory, semantic memory, working memory, perceptual speed, visuospatial and, global cognitive scores (GCS), as reported previously [10, 11]. Average time from the last clinical evaluation to death was ~8 months. Braak staging [12], Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [13], and the National Institute on Aging (NIA)-Reagan [14]

criteria were applied to the RROS subjects as described elsewhere [10, 11, 15, 16]. Five MCI cases were amnestic MCI (aMCI). Cases with other pathologies (e.g., stroke, Parkinson's disease, Lewy body or vascular dementia and hippocampal sclerosis) were excluded. Clinical, demographic and neuropathological characteristics of the NCI, MCI and mAD RROS cases are presented in Table 1.

A board-certified neuropathologist or trained technician blinded to all clinical data counted total number of neuritic plaques (NPs), diffuse plaques (DPs), and neurofibrillary tangles (NFTs) in one square mm area (100x magnification) per cortical region from the RROS cases examined [15, 17, 18]. Bielschowsky stain was used to visualize and count NPs, DPs and NFTs. Immunohistochemistry using an antibody against Aβ (4G8, 1:9000, Covance, WI) was used to visualize and measure Aβ density as previously described [19]. The samples from the RADC were evaluated only for NFTs using thioflavin-S to determine Braak staging.

Quantitative immunoblotting

Frontal cortex and hippocampal tissue were dissected free of white matter on dry ice and frozen at -80 °C. Samples were homogenized (150 mg/ml) in a phosphate buffered containing protease inhibitors (Sigma, St. Louis, MO). A rabbit polyclonal antibody against the C terminal amino acids 536–565 of Human PION was used to detect the full length 98 kDa and the 16 kDa GSAP forms (1:500, Abcam ab113024, Cambridge, MA). Loading control was a β-tubulin antibody (1:1000, Millipore, Billerica, MA) [15, 20].

Briefly, proteins were denatured in SDS loading buffer to a final concentration of 5 mg/ml. Proteins (50 μg/sample) were separated by 4–20% SDS-PAGE (Lonza, Rockland, ME) and electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore) [20]. Membranes were blocked in Tris-buffered saline (TBS)/ 0.05% Tween-20/ 5% milk (1 h) at room temperature (RT). GSAP antibody was added to blocking buffer and membranes were incubated overnight (4°C), washed, incubated at RT (1 h) with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:8000, Bio-Rad, Hercules, CA) or goat-anti rabbit IgG secondary antibody (1:5000, Bio-Rad, Hercules, CA), visualized by chemiluminescence on a Kodak Image Station 440CF (Perkin- Elmer, Wellesley, MA) and the 98 and 16 kDa GSAP bands were quantified across clinical groups using Kodak 1. Protein signals were normalized to β-tubulin and analyzed in three independent experiments [20].

Statistical Analysis

The Kruskal-Wallis, Mann-Whitney, Chi-square test were used to assess differences across clinical groups follow by a Dunn's post hoc test for multiple comparisons. Spearman rank was used to correlate the data. Statistical significance was set at $p < 0.05$ (two-tailed) and measurements were graphically represented using a Sigma Plot 12.5 software.

RESULTS

Case demographics

RROS cases did not differ by age, gender, education, post-mortem interval (PMI) or brain weight (see Table 1). There were no significant differences in the number of cases carrying the APOE ε4 allele between clinical groups. MMSE, perceptual speed and GCS were significantly ($p < 0.001$) lower in mAD compared to MCI and NCI, while differences between the latter two groups did not reach significance. Episodic memory scores were significantly lower for mAD compared to NCI and MCI, while MCI scores were lower than NCI ($p < 0.001$). No differences in semantic memory and working memory scores were found across the RROS clinical groups examined. NCI subjects displayed a visuospatial z-score that was significantly higher than the mAD cohort ($p < 0.04$). Braak score, CERAD and NIA Reagan diagnosis were not significantly different among the RROS clinical groups. Evaluation of Braak scores revealed that 50% of NCI and MCI cases were categorized as stages III-IV, while mAD displayed stages IV-V.

Demographics for sAD (n=13) cases revealed an average age at death of 78.46 ± 4.66 yr (range 71–86 yr), PMI of 5.21 ± 2.41 h (range 2–12 h), brain weight of 1,137.91 ± 150.80 g (range 940–1375 g), MMSE of 2.54 ± 4.36 (range 0–13), Braak stages of IV-V and 61% were female. This group lacked the extensive cognitive domain and neuropathological examination described for the RROS cases.

GSAP frontal cortex and hippocampal protein levels

Group-wise comparisons for frontal cortex and hippocampal proteins are shown in Table 2. Significant differences were observed for both GSAP 16 kDa and 98 kDa levels in the frontal cortex (Fig. 1A-C), but not in the hippocampus (Fig. S1A-C). Frontal cortex 16 kDa GSAP levels were significantly decreased in sAD compared to mAD and MCI, but not to NCI (Fig. 1A, B; Kruskal Wallis, $p = 0.01$). There were no significant differences between the mAD, MCI and NCI groups. Conversely, frontal cortex 98 kDa GSAP levels were significantly higher in sAD compared to NCI and mAD (Fig 1A, C; Kruskal Wallis, $p = 0.002$), but not to the MCI group. The mAD, NCI and MCI groups were not significantly different from each other. The ratio of frontal cortex 16 kDa to 98 kDa GSAP was significantly lower in sAD compared to the other three clinical groups (Fig. 1D; Kruskal-Wallis, $p < 0.001$), while the sAD hippocampal ratio was significantly decreased compared to MCI (Fig. S1D; Kruskal-Wallis, $p = 0.002$). A sub-analysis comparing cases with low (I-III) and high (IV-V) Braak scores in each clinical group revealed a significant decrease in hippocampal 16kDa protein leels, but not 98kDa, within high compared to those with low Braak scores (Mann-Whitney, $p < 0.05$) in NCI, but not in the MCI or mAD groups. However, a comparison across clinical groups using low and high Braak NCI subgroups did not reveal significant differences in hippocampal 16 kDa protein levels (Kruskal-Wallis, $p > 0.05$). No significant differences in frontal cortex or hippocampal 98 kDa and 16 kDa protein levels were observed between the amnestic and non-amnestic MCI cases (Mann-Whitney, $p > 0.05$).

Association of GSAP levels with clinical and pathological variables

Correlation analysis between GSAP proteins and the cognitive measures evaluated across clinical groups are shown in Table S1. Frontal cortex and hippocampal 16 kDa GSAP levels were not associated with Aβ, NFT, cognitive measures, or neuropathological criteria (Spearman correlation, $p > 0.05$). While cortical 98 kDa GSAP levels were moderately correlated with episodic memory (Spearman correlation, $r = -0.51$, $p = 0.003$), MMSE (Spearman correlation, $r = -0.41$, $p = 0.008$) and weakly with GCS (Spearman correlation, $r = -0.40$, $p = 0.02$). Hippocampal 98 kDa GSAP levels were only weakly correlated with MMSE values (Spearman correlation, $r = -0.40$, $p = 0.01$). Scatterplots of these associations are shown in Fig. S2. Furthermore, frontal and hippocampal 16 kDa and 98 kDa GSAP values did not correlate with cognitive measures for MMSE, GCS and episodic memory in each clinical group ($p > 0.05$). Whereas, hippocampal 16 kDa (Spearman correlation, $r = -0.9$, $p< 0.00001$) and 98 kDa (Spearman correlation, $r = -0.8$, 0.009) protein values were negatively associated with Braak scores in NCI cases.

DISCUSSION

GSAP has been identified as a potential novel therapeutic target for AD based on inhibition of its activity and reduction of Aβ levels without affecting notch cleavage of γ-secretase [5, 6]. However, virtually nothing is known about brain GSAP protein levels and their association with cognition and neuropathology during the onset of AD. Here we report the first evaluation of brain GSAP protein levels derived from tissue obtained from subjects with a pre-mortem clinical diagnosis of NCI, MCI, mAD and sAD. We found that both, the precursor and active forms of GSAP were altered in the frontal cortex but not the hippocampus during the clinical progression of AD. These findings suggest that GSAP protein levels are affected differently depending upon brain region, likely related to AD-pathology prevalence or other neurodegenerative processes.

In the present study, we found a significant upregulation of full-length (98 kDa) GSAP, which co-occurred with a downregulation of the active form (16 kDa) of this protein in the neocortex in sAD cases. Conversely, a recent report using frontal cortex from a small number of individuals (7 AD and 4 controls) showed an increase in the levels of the active, but not full-length GSAP [22]. The discrepancies between these findings and ours may be related to differences in methodologies, study design and/or cohorts examined. Interestingly, levels of the full-length 98 kDa and active 16 kDa GSAP forms were significantly increased in the frontal cortex of patients with Down Syndrome (DS) compared age-matched controls [9]. The discrepancy in 16 kDa GSAP levels between DS and AD (present findings) could be related to the presence of an extra copy of the APP gene in DS that might stimulate the expression of GSAP or other confounding components such as transcription factors [9, 22]. Data indicate that formation of the 16 kDa GSAP active form depends on caspase-3 [9], an apoptotic marker that is elevated and highly expressed in neurofibrillary tangles and plaques, in both human AD and animal models of this disease [21]. Although previous investigations indicate that 16 kDa GSAP levels are associated with Aβ deposition [5, 6], here we found that GSAP protein levels did not correlate with amyloid and NFT scores either in the frontal cortex or hippocampus across clinical groups. Nevertheless neocortical full-length GSAP

levels did correlate with MMSE and episodic memory revealing a potential role of the full length but not the active form of GSAP in cognitive impairment in AD. Interestingly, we found that NCI cases with high, but not low Braak scores showed a decrease in the level of 16 kDa GSAP in the hippocampus. Moreover, in the NCI cases we found that 16 kDa GSAP levels correlated with Braak pathology, suggesting that the active form of this protein is dysregulated in the hippocampus in elderly individuals with high NFT Braak scores, but without cognitive impairment.

In summary, our findings indicate that protein levels for the full length and the active forms of GSAP are differentially altered in the frontal cortex compared to the hippocampus during the progression of AD. Neocortical full-length GSAP increases while the active form decreases in sAD, whereas both are preserved in the hippocampus across clinical groups. In vitro and in vivo studies suggest that 16 kDa GSAP inhibition reduces amyloid deposition [5, 6]. However, we found a decrease in 16 kDa and an increase in 98 kDa GSAP in severe AD despite high amyloid deposits. Taking together these findings suggest that GSAP does not play a significant role in Aβ production early in the disease process. Further studies are required to investigate the interaction between the different forms of GSAP, Aβ production and cognition in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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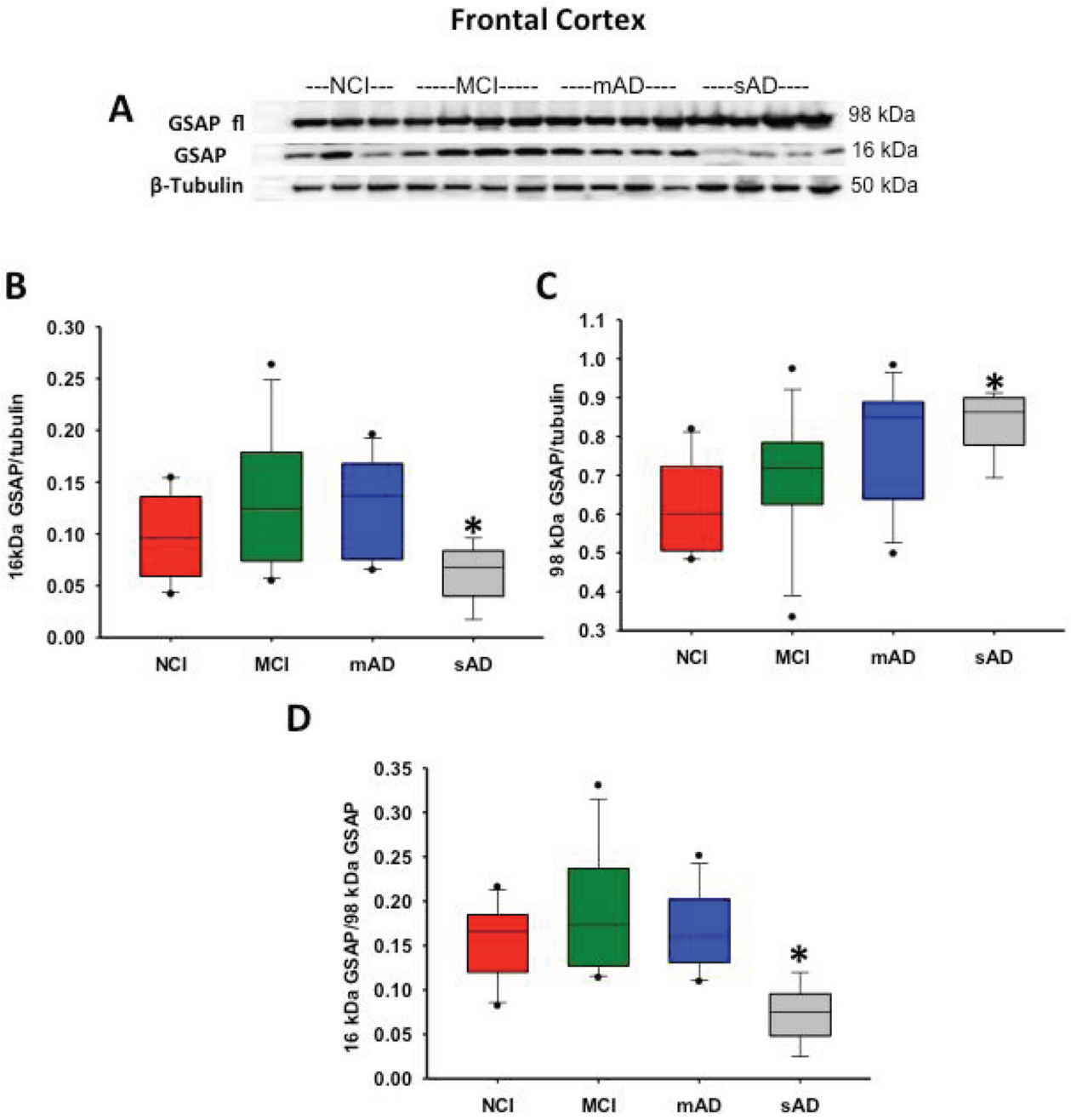

Figure 1.

Representative immunoblots (**A**) and box plots (**C**-**D**) showing frontal cortex 98 kDa and 16 kDa GSAP protein levels from cases with a premortem clinical diagnosis of NCI, MCI, mAD and sAD. β-tubulin was used to normalize the immunoreactive signal obtained by densitometry. (**A**). Western blots showing differential reactivity between the 98kDa and 16 kDa GSAP protein compared to β-tubulin across clinical groups. (**B**) 16 kDa GSAP levels were significantly reduced in the frontal cortex of sAD compared MCI and mAD (*p = 0.01) cases, whereas 98kDa levels (**C**) were also significantly higher compared to NCI and mAD (*p = 0.002). (**D**) The ratio of 16 kDa to 98 kDa GSAP protein levels was significantly

higher in sAD compared to NCI, MCI and mAD (*p < 0.001). NCI, non-cognitive impairment, MCI, mild cognitive impairment, mAD, mild to moderate AD, sAD, severe AD. Black circles in the box plots indicate outliers. Black lines in the box blots indicate median and whiskers indicate maximum and minimum deviation.

Table 1

Demographic, cognitive, and neuropathological characteristics by clinical groups

		NCI (N=14)	MCI (N=16)	mAD (N=13)	Total (N = 43)	Overall p-value	Group-wise comparisons
Age (years) at death	Mean ± SD	85.66 ± 4.76	86.74 ± 4.86	89.37 ± 6.13	87.10 ± 5.25	0.21 *	-----
	Range	(78 – 94)	(79 – 95)	(76 – 100)	(76 – 100)		
Number of males (%)		6 (43)	6 (37)	4 (31)	16 (37)	0.94 †	-----
Years of education	Mean ± SD	17.14 ± 3.87	17.88 ± 3.39	18.31 ± 2.99	18.07 ± 3.47	0.44 *	-----
	Range	(10 – 23)	(10 – 25)	(16 – 26)	(10 – 26)		
Number with APOE *ε*4 allele (%)		0 (0)	5 (31)	2 (15)	7 (16)	0.18 †	**NCI < MCI**
MMSE	Mean ± SD	27.92 ± 1.50	27.00 ± 2.63	21.46 ± 4.70	25.63 ± 4.16	0.001 *	**NCI, MCI > mAD**
	Range	(26 – 30)	(22 – 30)	(14 – 28)	(14 – 30)		
GCS	Mean ± SD	−0.02 ± 0.28	−0.43 ± 0.35	−1.18 ± 0.58	−0.53 ± 0.61	<0.001 *	**NCI, MCI > mAD**
	Range	(−0.41 – 0.42)	(−1.20 – 0.40)	(−2.07 – 0.10)	(−2.07 – 0.42)		
Episodic memory z-score	Mean ± SD	0.24 ± 0.43	−0.20 ± 0.56	−1.62 ± 0.75	−0.37 ± 0.90	<0.001 *	**NCI > MCI > mAD**
	Range	(−0.61 – 0.91)	(−1.20 – 0.03)	(−2.07 – 0.10)	(−3.11 – 1.31)		
Semantic memory z-score	Mean ± SD	−0.18 ± 0.72	−0.38 ± 0.59	−0.77 ± 0.88	−0.43 ± 0.75	0.10 *	-----
	Range	(−1.44 – 0.40)	(−1.57 – 0.36)	(−2.95 – 0.15)	(−2.95 – 0.40)		
Working memory z-score	Mean ± SD	−0.08 ± 0.45	−0.27 ± 0.63	−0.66 ± 0.68	−0.33 ± 0.62	0.09 *	-----
	Range	(−0.96 – 0.47)	(−1.31 – 1.08)	(−1.77 – 0.56)	(−1.77 – 1.08)		
Perceptual speed z-score	Mean ± SD	−0.66 ± 0.69	−0.94 ± 0.58	−2.29 ± 0.83	−1.26 ± 0.97	<0.001 *	**NCI, MCI > mAD**
	Range	(−1.62 – 0.45)	(−2.08 – (−.04))	(−3.38 – (−1.04))	(−3.38 – 0.45)		
Visuospatial z-score	Mean ± SD	−0.40 ± 0.64	−0.77 ± 0.66	−1.05 ± 0.64	−0.73 ± 0.68	0.04 *	**NCI>mAD**
	Range	(−1.25 – 0.74)	(−1.67 – 0.44)	(−2.12 – 0.29)	(−2.12 – 0.74)		
Post-mortem interval (hours)	Mean ± SD	5.74 ± 2.13	5.45 ± 1.72	4.77 ± 2.55	5.34 ± 2.12	0.23 *	-----
	Range	(1 – 9)	(2 – 9)	(1 – 10)	(1 – 10)		

		NCI (N=14)	MCI (N=16)	mAD (N=13)	Total (N = 43)	Overall p-value	Group-wise comparisons
Brain weight (g)	Mean ± SD	1208.36 ± 104.92	1179.25 ± 129.19	1138.46 ± 104.09	1176.40 ± 115.12	0.26 *	-----
	Range	(1000 – 1400)	(990 – 1480)	(962 – 1320)	(962 – 1480)		
Distribution of Braak scores	0	0	0	0	0	0.09 *	-----
	I/II	2	3	3	8		
	III/IV	11	11	7	29		
	V/VI	1	2	3	6		
NIA Reagan (likelihood of AD)	No AD	0	0	0	0	0.09 *	-----
	Low	8	9	3	20		
	Intermediate	6	5	7	18		
	High	0	2	3	5		
CERAD	No AD	5	6	1	12	0.06 *	-----
	Possible	3	2	1	6		
	Probable	4	5	6	15		
	Definite	2	3	5	1		

* Kruskal-Wallis test with Dunn's test for pair-wise comparisons;

† Chi-square test; severe AD cases not shown in table.

Table 2

Summary of GSAP protein levels in the frontal cortex and hippocampus by clinical groups

	NCI	MCI	mAD	sAD	p-value	Group-wise Comparisons
16kDa GSAP FC	0.10 ± 0.04 (0.04, 0.15)	0.13 ± 0.04 (0.05, 0.26)	0.13 ± 0.04 (0.07, 0.20)	0.06 ±0.03 (0.02, 0.10)	0.01	MCI, mAD > sAD
98kDa GSAP FC	0.60 ± 0.12 (0.48, 0.82)	0.70 ± 0.16 (0.33, 0.97)	0.80 ± 0.14 (0.50, 0.98)	0.84 ± 0.07 (0.69, 0.91)	0.002	NCI, mAD < sAD
16 kDa/98 kDa GSAP FC	0.15 ± 0.041 (0.08, 0.21)	0.19 ± 0.07 (0.11, 0.33)	0.16 ± 0.04 (0.11, 0.25)	0.07 ± 0.03 (0.02, 0.12)	< 0.001	NCI, MCI, mAD > sAD
16 kDa GSAP HP	0.14 ± 0.08 (0.06, 0.28)	0.18 ± 0.05 (0.07, 0.25)	0.12 ± 0.05 (0.05, 0.20)	0.12 ± 0.03 (0.09, 0.180)	0.07	--
98 kDa GSAP HP	0.59 ± 0.19 (0.38, 1.02)	0.60 ± 0.12 (0.34, 0.74)	0.55 ±0.18 (0.34, 0.83)	0.72 ±0.16 (0.46, 0.88)	0.24	--
16 kDa/98 kDa GSAP HP	0.23 ±0.09 (0.12, 0.42)	0.30 ± 0.08 (0.20, 0.46)	0.21 ± 0.04 (0.15, 0.26)	0.16 ± 0.04 (0.11, 0.21)	0.002	MCI > sAD

Mean ± SD (Range); p-values were derived from the Kruskal-Wallis follow of a Dunn's test for multiple comparisons.

FC: frontal cortex; HP: hippocampus.