



Neurogranin in Alzheimer's disease and ageing: A human post-mortem study

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ABSTRACT

Neurogranin (Ng), a post-synaptic protein involved in memory formation, has been investigated as a biomarker in the cerebrospinal fluid (CSF) in Alzheimer's disease (AD) and ageing. CSF Ng levels are elevated in AD relative to healthy controls and correlate with cognition; however, few studies have focused on Ng abundance in the brain. Synapse loss in the brain correlates closely with cognitive decline in AD making synaptic biomarkers potentially important for tracking disease progression, but the links between synaptic protein changes in CSF and brain remain incompletely understood. In the current study, Ng abundance was examined in post-mortem human brain tissue across AD, healthy ageing (HA), and mid-life (ML) cohorts. Ng levels were quantified in three brain regions associated with cognitive change found during ageing and neurodegenerative diseases, namely the middle temporal gyrus, primary visual cortex and the posterior hippocampus using immunohistochemistry. To support immunohistochemical analysis, total homogenate and biochemically enriched synaptic fractions from available temporal gyrus tissues were examined by immunoblot. Finally, we examined whether Ng is associated with lifetime cognitive ageing. Ng levels were significantly reduced in AD relative to HA and ML cases across all regions. Additionally Ng was significantly reduced in HA in comparison to ML in the primary visual cortex. Immunoblotting confirms reduced Ng levels in AD cases supporting immunohistochemical results. Interestingly, there was also a significant reduction of synapse-associated Ng in our group who had lifetime cognitive decline in comparison to the group with lifetime cognitive resilience indicating loss of neurogranin in remaining synapses during ageing is associated with cognitive decline. Our findings indicate that increases in CSF Ng reflect loss of brain neurogranin and support the use of CSF Ng as a biomarker of AD and potentially of cognitive decline in healthy ageing.

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1. Introduction

By the year 2050, 2 billion people are expected to be over the age of 60 and the number of people living dementia is projected to reach 152 million (World Health Organisation, 2020; World Health Organisation, 2018). An ageing population is associated with several challenges, including age-related cognitive decline and increased incidence of dementia, both of which can have a significant physical, psychological, social, and economical impact on individuals and their carers (Bárríos et al., 2013). Biomarkers associated with age-related cognitive decline and cognitive decline due to dementia will assist in understanding underlying aetiologies and may eventually lead to the development of effective interventions.

In AD the strongest pathological correlate of cognitive decline is synapse loss (de Wilde et al., 2016; DeKosky and Scheff, 1990), prompting the investigation of synaptic proteins as markers associated with cognitive decline. Increasingly, the post-synaptic protein neurogranin (Ng) has been investigated as a potential marker associated with cognition. Ng (also known as RC3, p17, or BICKS) is a 78 amino acid polypeptide (de Arrieta et al., 1997) thought to play a critical role in long-term potentiation (LTP). It was first identified as a substrate of protein kinase C (PKC) in bovine brain (Baudier et al., 1989) and was later characterised in rodents (Huang et al., 1993; Watson et al., 1990). In murine models, Ng concentration in hippocampal regions decrease with age and are implicated in central nervous system dysfunction (Mons et al., 2001). Behavioural studies of *Nrgn* knockout (*Nrgn* KO) mice have identified phenotypes associated with hyperactivity, deficits in spatial learning, impaired sociability, motor dysfunction and altered anxiety (Nakajima et al., 2021). In CamKII-TetOp25 mouse models (inducible model of severe synaptic loss and brain atrophy) Ng levels increased in CSF when neurodegeneration was induced whilst they decreased in brain supporting CSF Ng as a biomarker of synaptic degeneration (Höglund et al., 2020). Levels of Ng are reduced in familial and sporadic AD brain tissue (Kvartsberg et al., 2019), but the levels of this protein in remaining synapses in AD have not been examined. There is some evidence that Ng in CSF is associated with cognitive decline in the absence of dementia (Casaletto et al., 2017) but the relationship between brain Ng and cognitive decline in ageing remains unknown.

Ng has a predicted mass of 7.5 kDa, although monomers have been found to migrate with an apparent mass of 15–19 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Díez-Guerra, 2010). Ng is an abundant protein across the brain and is primarily localised in dendritic spines of pyramidal neurons (Watson et al., 1992). It contains an IQ motif (Baudier et al., 1991) which allows it to bind calmodulin (CaM) in the absence of calcium (Ca^{2+}) (Kubota et al., 2007). Ng sequesters CaM at dendritic spines and can release it when intracellular Ca^{2+} concentrations increase, thus regulating the availability of CaM, and therefore Ca^{2+} / CaM signalling (Díez-Guerra, 2010; Petersen and Gerges, 2015). This has downstream effects on the activity of several Ca^{2+} / CaM-dependent enzymes which are required for long-term potentiation and long-term depression (Huang, 2004).

Its dendritic spine, localisation and abundance may make Ng a suitable marker for synapse loss. It is well established that cerebrospinal fluid (CSF) levels of Ng are increased in AD and MCI relative to controls (Kester et al., 2015; Kvartsberg et al., 2015; De Vos et al., 2015; Thorsell et al., 2010) and can be used to discriminate between AD and other neurodegenerative diseases (Willemse et al., 2018; Portelius et al., 2018). Fewer studies, however, have investigated Ng levels in post-mortem human brain samples. In early-onset AD (EAD), Ng is reduced in both frontal cortex and hippocampus compared to both controls and late-onset AD cases (Reddy et al., 2005a; Davidsson and Blennow, 1998). Furthermore, an increased ratio of peptides to full-length Ng has been reported in AD parietal and temporal cortex relative to controls (Kvartsberg et al., 2019), suggesting proteolytic processing of full-length Ng during synapse degeneration. In a large sample of human post-mortem samples, Ng (*NRGN*) gene expression has been found to

negatively correlate with amyloid and tau pathology in the perirhinal cortex, functional clinical dementia rating (CDR) scores, and is associated with a neuropathological diagnosis of AD (Sun et al., 2021). Despite several studies examining Ng protein levels in AD, at present there are no known studies examining these differences using immunohistochemistry methods. Further, previous research adopting the use of western blots to examine relative protein levels have all used total homogenate preparations of brain tissue. Synaptoneurosome preparations, which consist of isolated synaptic compartments (Tai et al., 2012), may provide further insight into relative Ng levels associated with synapses.

In the current study, we examined Ng levels across mid-life, aged control subjects without dementia, and AD cohorts. Three brain regions were selected based on their roles in learning and memory function and their involvement in or resilience to neurodegeneration in AD (Keller, 2006; Cui et al., 2007; Lee et al., 2022), namely middle/inferior temporal gyrus (BA20/21); primary visual cortex (BA17) and posterior hippocampus (HC). We included immunohistochemical analysis and synaptoneurosome tissue preparations for completeness. Finally we investigated whether Ng levels are associated with.

lifetime cognitive ageing differences in a unique cohort of non-demented healthy agers.

2. Methods

2.1. Post-mortem samples

Use of human tissue for post-mortem studies has been reviewed and approved by the Academic and Clinical Central Office for Research and Development medical research ethics committee (approval 15-HV-016) and the Edinburgh Brain Bank (research ethics committee approval 16/ES/0084). In total, tissue from 38 donors was used in the current study (see Table 1/ 2 for individual subject characteristics/ demographics). Donors were either mid-life controls with no known neurological conditions (ML), healthy agers without dementia (HA) (subset from the Lothian Birth Cohort 1936) (Deary et al., 2007) and AD cases with confirmed clinical and neuropathological diagnosis of Alzheimer's Disease (AD).

2.2. Cognitive categorising of healthy agers

Pre-morbid longitudinal cognitive data were available for HA donors, which was calculated from the Moray House Test No. 12 (Scottish Council for Research in Education, 1933), a measure of general intelligence which was administered to school children aged ~C;11 in Scotland in 1947. These participants (wave 1, $n = 1091$) were followed-up decades later, aged ~C;70 years, with triennial assessments (mean ages ~C;70, 73, 76) (Deary et al., 2007; Taylor et al., 2018). Longitudinal MHT scores were used to sub-categorise donors from our healthy agers.

This classification was achieved by plotting age-adjusted MHT scores from age 11 against the mean of older-age adjusted MHT scores from ages 70 and 76 ($n = 641$) (King et al., 2022). Donors of post-mortem tissue were plotted on this lifetime cognitive plot, where samples above the regression line were defined as lifetime cognitive resilient (LCR) and those below lifetime cognitive decline (LCD) (King et al., 2022) (see Table 3 for individual HA cognitive and education status). TaqMan technology was used to identify *APOE* e4 carriers. Status was determined according to genotyping on the two polymorphic sites (rs7412 and rs429358) that account for e2-e4 alleles (Wenham et al., 1991) Allelic status for the purposes of the current analyses was a binary variable denoting e4 present/absent.

2.3. Synaptoneurosome preparations

Total brain homogenates and synaptoneurosome were prepared according to Tai et al. 2012 (Tai et al., 2012). 200 mg of freshly frozen

Table 1

Post-mortem sample subject characteristics.

BBN number	Group	Method	Age	PMI	Sex	Braak stage	Thal Stage	Cognitive status
001.28406	HA	WB	79	72	Male	2	2	LCD
001.32577	HA	IHC	81	74	Male	2	3	LCD
001.28793	HA	WB	79	72	Female	2	1	Unavailable
001.28794	HA	IHC	79	61	Female	1	0	LCR
001.26495	HA	WB	78	39	Male	1	1	LCD
001.28797	HA	IHC	79	57	Male	0	0	LCR
001.31495	HA	WB	81	38	Male	6	4	LCR
001.28402	HA	IHC	79	49	Male	1	2	LCD
001.19686	HA	WB	77	75	Female	1	1	LCD
001.34131	HA	IHC	82	95	Male	4	3	LCD
001.29082	HA	WB	79	80	Female	3	5	LCR
001.29086	HA	IHC	79	68	Female	0	1	LCR
001.35215	HA	WB	82	40	Male	1	0	LCR
001.35549	HA	IHC	82	56	Male	1	1	LCR
001.36135	HA	IHC	84	30	Female	2	1	LCR
001.24479	ML	WB	46	76	Female	0	0	CON
001.29906	ML	IHC	51	52	Male	Unavailable	Unavailable	CON
001.33613	ML	WB	46	99	Female	0	0	CON
001.30169	ML	IHC	48	58	Male	Unavailable	Unavailable	CON
001.34244	ML	WB	49	69	Female	0	0	CON
001.29466	ML	IHC	39	76	Male	0	0	CON
001.24342	ML	WB	33	47	Male	0	0	CON
001.28792	ML	IHC	58	51	Male	Unavailable	Unavailable	CON
001.30972	ML	WB	34	99	Male	0	0	CON
001.26976	ML	IHC	19	101	Male	0	0	CON
001.36066	AD	WB	94	29	Male	6	5	dementia
001.29695	AD	IHC	86	72	Male	6	5	dementia
001.35183	AD	WB	74	75	Male	6	5	dementia
001.30142	AD	IHC	88	112	Female	6	5	dementia
001.29135	AD	WB	90	73	Male	6	3	dementia
001.35564	AD	IHC	90	52	Female	6	5	dementia
001.30883	AD	WB	61	69	Female	6	5	dementia
001.29521	AD	IHC	95	96	Male	6	5	dementia
001.30973	AD	WB	89	96	Female	6	5	dementia
001.36328	AD	IHC	71	81	Male	6	5	dementia
001.36346	AD	WB	90	60	Female	6	5	dementia
001.35535	AD	IHC	83	95	Female	6	4	dementia
001.26718	AD	WB	78	74	Male	6	5	dementia

Abbreviations: BBN - brain bank number; AD - Alzheimer's disease; WB - Western blot; IHC -Immunohistochemistry; HA, healthy ager; ML, mid-life; LCD, lifetime cognitive decline; LCR, lifetime cognitive reserve; CON – control with no neurological or psychiatric diagnoses; PMI, post-mortem interval (hours).

human brain tissue (BA20/21) was homogenised in 1 mL buffer (25 mM/L HEPES pH 7.5, 120 mM/L NaCl, 5 mM/L KCl 1 mM/L MgCl₂, 2 mM/L CaCl₂), with protease inhibitors (Roche complete mini) and phosphatase inhibitors (Millipore). The homogenate was passed through an 80 µm nylon filter (Millipore) and a 300 µL aliquot was saved and mixed with buffer (100 mM/L Tris-HCl pH 7.6, 4% SDS, protease inhibitor cocktail EDTA-free 100× Thermo Fisher) to prepare the crude total homogenate. The remainder of the homogenate was passed through a 5 µm filter (Millipore) then centrifuged at 1000 xg for 5 min.

The supernatant was discarded and the pellet was washed with buffer and centrifuged again yielding the synaptoneurosome pellet. Successful enrichment of synapses in synaptoneurosome has been shown previously in the lab (King et al., 2022).

2.4. Western blotting

Protein concentrations were determined using a protein assay (Thermo Fisher). 20 µg of protein per sample was electrophoresed in

4–12% Bis-Tris polyacrylamide gels (Invitrogen). Proteins were electro-transferred to nitrocellulose membranes (Thermo Fisher) using the iBlot™ Dry Blotting system (Invitrogen). Revert 700 Total Protein Stain was used to quantify total protein (Li-Cor). Membranes were incubated in blocking buffer, then incubated with relevant primary antibodies in blocking buffer. Two monoclonal Ng antibodies were used which bind to different epitopes of the C-terminus of Ng, both of concentrations 1 mg/mL (Fig. 1A), namely Ng2 (1:500, University of Gothenburg) and Ng36 (1:500, University of Gothenburg). The generation of Ng2 and Ng36 has been described in detail previously (Kvartsberg et al., 2019; Kvartsberg et al., 2015). Both antibodies were selected for this study based on previous characterisation profiling involving epitope mapping, mass spectrometry and western blotting techniques (Kvartsberg et al., 2019; Kvartsberg et al., 2015). These antibodies are also used in the Roche NeuroToolKit for Ng (van Hulle et al., 2021) and are among the best characterised antibodies in the world (Portelius et al., 2018). Membranes with the same cases were run separately with Ng2 and Ng36. Membranes were washed and incubated with secondary antibodies (1:5000, Li-Cor), rinsed and imaged using the Odyssey Imaging system, and analysed using Open Image Studio. Target proteins of interest were

normalised to total protein. Results are presented from a single run.

2.5. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections of 4 µm thickness from BA20/21, BA17, and hippocampus were processed for immuno-histochemistry using the Leica Novolink Polymer Detection Kit (RE7280-K). Serial sections were stained for Ng (Ng2, 1:500). The chromogen used for visualisation was 3,3'-diaminobenzidine (DAB) with 0.05% hydrogen peroxide as substrate. Tissue was counterstained with haematoxylin for 30 s to visualise cell nuclei. Slides were mounted using DPX (M192).

2.6. Thresholding and Ng expression quantification

Slides were visualised using a ZEISS Imager.Z2 stereology microscope and researchers were blinded to groups. All six layers of the grey matter were included in the analysis using the MBF Biosciences Stereo Investigator 2019 software. Cortical grey matter was outlined at 1.5× objective magnification and tile scans were obtained at 5× for

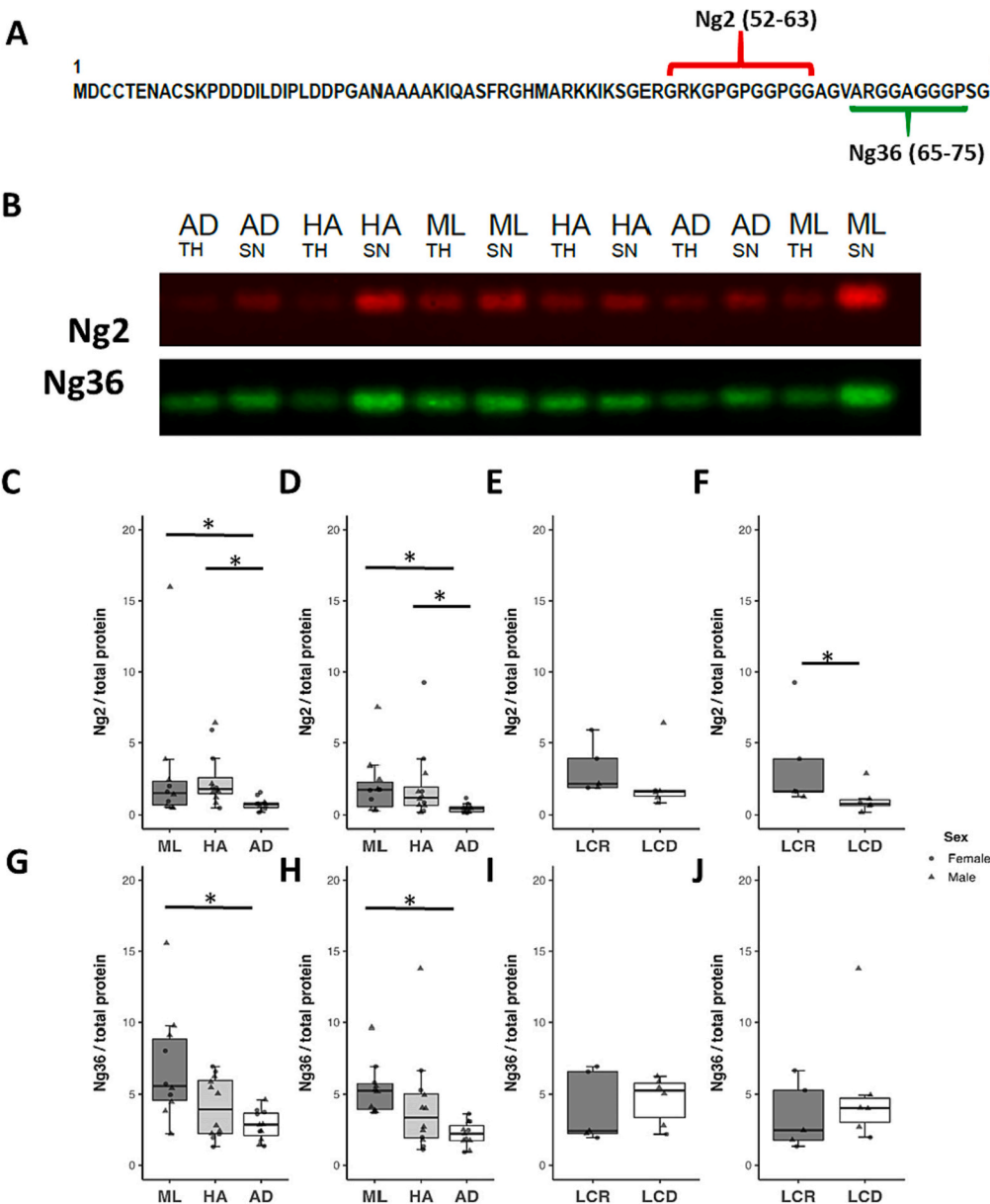


Fig. 1. Neurogranin protein levels are decreased in B20/21 synapses in AD and poorer cognitive cohorts. A Illustration of epitope location of both Ng2 (red) and Ng36 (green) antibodies. B Representative immunoblots using both antibodies across ML, HA and AD cases and total homogenate (TH) and synaptoneurosomes (SN) preparations. C Total homogenate and D Synaptoneurosomes Ng2 protein levels plotted across ML, HA and AD. E Total homogenate and F Synaptoneurosomes Ng2 protein levels plotted between cognitive split, lifetime cognitive resilient (LCR) or lifetime cognitive decline (LCD) cohorts. G–J Same as C–F but with Ng36 antibody. For boxplots, each data point represents an individual raw Ng/total protein value. Statistical analyses were conducted using ANOVA for plots C,D,G,H (Ng ~C; Cohort) and *t*-tests for plots E,F,I,J (Ng ~C; Cognitive status). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantification. Ng levels were quantified using an in-built software algorithm which identifies immuno-labelled objects based on a colour and size threshold detailed in Tzioras et al., 2017 (Tzioras et al., 2017). Objects smaller than 100 μm^2 was excluded in the Ng analysis as they did not represent true immunostaining. Outlined objects were then transferred to Neurolucida Explorer which calculated the total area of the region of interest and the summed area of the outlined objects, producing a percentage of Ng expression.

2.7. Statistical analysis

R Studio (version 4.1.2) was used for all data analysis (R script outputs and associated files are included as supplementary files namely Ng script word, Ng IHC data and Ng WB data). Data were tested for normality by inspection of histograms and Shapiro-Wilk tests. To meet the assumption of normality, Ng data were transformed using the Tukey Ladder of Power method. This resulted in Ng expression data being log transformed and Ng western blot data being transformed by the reciprocal squared. Linear mixed effects models were used to examine group differences in Ng across BA17, BA20/21, and the posterior hippocampus. Group, brain region, a group * brain region interaction, APOE status, sex and PMI were entered as fixed effects, while case number was entered as a random effect with random intercepts. The overall significance of fixed effects was examined using an F test. Analysis of variance (ANOVA) was conducted to examine group differences in relative Ng levels between the three groups with the same covariates as above. *Post-hoc* analysis was conducted using Tukey's test for *post-hoc* analysis. Differences between healthy agers with lifetime cognitive decline (LCD) and those with lifetime cognitive resilience (LCR) was examined using independent samples *t*-tests. Significance was defined where $p < 0.05$.

3. Results

3.1. Neurogranin is decreased in BA20/21 synaptic compartments in Alzheimer's disease and people with lifetime cognitive decline in the absence of dementia

Thirty-three cases were used for western blot analyses of brain tissue: 10 mid-life (ML), 12 healthy-agers (HA), and 11 CE using two antibodies specific for neurogranin Ng2 and Ng36 (Fig. 1A). Demographics are provided in Table 2. Between the three groups ML, HA, AD, there were no significant differences in sex ($\chi^2 = 0.56$, $df = 2$, $p = 0.7$) or in PMI ($F(2, 30) = 0.68$, $p = 0.5$). There was a significant difference in age ($F(2, 30) = 70.81$, $p = 4.35e-12$), with ML subjects being significantly younger than both AD cases ($p = 0.0001$) and HA cases ($p = 0.0001$), although there was no significant difference between AD and HA cases ($p = 0.19$). Both antibodies worked well with immunoblotting and were complementary to each other (Fig. 1B). There was a modest but significant correlation between Ng2 and Ng36 in both total homogenate ($r = 0.48$, $p = 0.005$) and in synaptoneurosome preparations ($r = 0.40$, $p = 0.02$). As shown in Fig. 1C-D, across the three groups, there was a significant difference in Ng2 levels in total homogenate ($F(2, 30) = 6.26$, $p = 0.005$) and in synaptoneurosome preparations ($F(2, 30) = 6.05$, $p = 0.006$). *Post-hoc* analyses of total homogenate data revealed significantly decreased Ng2 in AD cases relative to HA ($p = 0.005$) and ML ($p = 0.03$) cases. There were no significant differences between HA and ML cases. Similarly, in synaptoneurosome preparations, Ng2 was significantly decreased at the synapse in AD relative to HA cases ($p = 0.01$) and ML cases ($p = 0.01$).

Changes in Ng36 levels were less pronounced possibly relating to epitope binding/availability but trends support Ng2 results (Fig. 1G-H). There was a significant difference in both total homogenate ($F(2, 30) = 6.67$, $p = 0.004$) and synaptoneurosome preparations ($F(2, 30) = 8.03$, $p = 0.001$) across the three groups. *Post-hoc* analyses revealed significantly decreased Ng36 in AD cases compared to ML in both total

Table 2
Post-mortem demographics.

Variable	Western blot study			IHC study		
Cohort	HA ($n = 12$)	ML ($n = 10$)	AD ($n = 11$)	HA ($n = 15$)	ML ($n = 6$)	AD ($n = 7$)
Age (years)	79.3 (1.34)	42.30 (10.94)	84.36 (10.56)	80.0 (1.89)	45.5 (6.41)	87.0 (5.42)
Sex						
F	5 (41.7%)	3 (30.0%)	5 (45.5%)	6 (40.0%)	3 (50.0%)	3 (42.9%)
M	7 (58.3%)	7 (70.0%)	6 (54.5%)	9 (60.0%)	3 (50.0%)	4 (57.1%)
PMI (hours)	65 (16.56)	72.80 (20.51)	74.09 (22.25)	60.4 (18.5)	66.8 (19.1)	88.3 (15.4)
APOE genotype						
2/2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2/3	1 (8.3%)	0 (0%)	0 (0%)	3 (20%)	0 (0%)	0 (0%)
2/4	0 (0%)	1 (10%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3/3	8 (66.7%)	1 (10%)	0 (0%)	9 (60%)	3 (50.0%)	1 (14.3%)
3/4	3 (25%)	4 (40%)	6 (54.5%)	3 (20%)	3 (50.0%)	6 (85.7%)
4/4	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)
NA	0 (0%)	4 (40%)	4 (36.4%)	0 (0%)	0 (0%)	0 (0%)
Brain pH	6.08 (0.21)	6.31 (0.24)	6.08 (0.14)	6.06 (0.19)	6.40 (0.18)	6.19 (0.22)
Brain weight (g)	1355 (90.30)	1418.40(95.82)	1170 (141.82)	1340 (91.6)	1390 (102)	1260 (115)
Braak stage						
0	2 (16.67%)	10 (100%)	0 (0%)	2 (13.3%)	6 (100%)	0 (0%)
1	4 (33.33%)	0 (0%)	0 (0%)	6 (40.0%)	0 (0%)	0 (0%)
2	3 (25%)	0 (0%)	0 (0%)	4 (26.7%)	0 (0%)	0 (0%)
3	1 (8.33%)	0 (0%)	0 (0%)	1 (6.7%)	0 (0%)	0 (0%)
4	1 (8.33%)	0 (0%)	0 (0%)	1 (6.7%)	0 (0%)	0 (0%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
6	1 (8.33%)	0 (0%)	11 (100%)	1 (6.7%)	0 (0%)	7 (100%)
Thal phase						
0	2 (16.67%)	10 (100%)	0	3 (20%)	6 (100%)	0 (0%)
1	4 (33.33%)	0 (0%)	0	6 (40%)	0 (0%)	0 (0%)
2	2 (16.67%)	0 (0%)	0	2 (13.3%)	0 (0%)	0 (0%)
3	2 (16.67%)	0 (0%)	1 (9.09%)	2 (13.3%)	0 (0%)	1 (14.3%)
4	1 (8.33%)	0 (0%)	0	1 (6.7%)	0 (0%)	1 (14.3%)
5	1 (8.33%)	0 (0%)	10 (90.91%)	1 (6.7%)	0 (0%)	5 (71.4%)

Abbreviations: AD, Alzheimer's disease; HA, healthy ager; ML, mid-life; PMI, post-mortem interval.

Table 3

Healthy agers by cognitive and education status.

BBN Number	Cognitive status	Education level
001.28406	LCD	School-leaver
001.32577	LCD	Unknown
001.26495	LCD	School-leaver
001.28402	LCD	School-leaver
001.19686	LCD	School-leaver
001.34131	LCD	School-leaver
001.28797	LCR	Further education
001.31495	LCR	Further education
001.29082	LCR	School-leaver
001.29086	LCR	Further education
001.35215	LCR	Unknown
001.35549	LCR	School-leaver
001.36135	LCR	School-leaver
001.28794	LCR	School-leaver
001.28793	Unavailable	School-leaver

Abbreviations: BBN - brain bank number; LCD, lifetime cognitive decline; LCR, lifetime cognitive reserve.

homogenate ($p = 0.003$) and synaptoneurosome preparations ($p = 0.001$). There were no significant differences between AD and HA cases, or HA and ML cases.

When the HA sample was split by lifetime cognitive ageing, those with Lifetime cognitive decline (LCD) had significantly decreased synaptic Ng2 levels relative to those with lifetime cognitive resilience (LCR) ($p = 0.04$) (Fig. 1F). There were no significant differences in total homogenate preparations (Fig. 1E), nor in Ng36 between the two groups in either total homogenate or synaptoneurosome preparations (Fig. 1I-J).

3.2. Histological analyses confirm decreases in neurogranin protein from mid-life through to healthy ageing and Alzheimer's disease in multiple brain regions

Twenty-eight cases were included in the IHC study using 15 HA, 6 ML, and 7 CE cases. Ng2 was used for immunolabelling as produced reliable immunostaining and representative images are shown in Fig. 2A. Individual case details are provided in Table 1 and group characteristics are provided in Table 2. There were no significant differences between the cohorts in sex ($\chi^2 = 0.17$, $df = 2$, $p = 0.92$). There was a significant difference in age ($F(2, 25) = 191.0$, $p = 7.16e-16$); ML cases were significantly younger than AD ($p = 0.0001$) and HA cases ($p = 0.0001$), and AD cases were significantly older than HA cases ($p = 0.003$). There was also a significant difference in PMI hours ($F(2, 25) = 5.83$, $p = 0.008$), with AD cases having a significantly longer PMI than HA cases ($p = 0.006$). As shown in Fig. 2B there is a significant association between cohort and Ng ($F(2, 21) = 19.12$, $p = 1.86e-05$). Post-hoc analyses revealed Ng levels were significantly higher in ML cases relative to AD in BA20/21 ($p = 0.0008$), BA17 ($p = 0.0001$) and hippocampus ($p = 0.0003$). HA cases had significantly higher neurogranin levels relative to AD cases in BA20/21 ($p = 0.04$), BA17 ($p = 0.01$) and hippocampus ($p = 0.02$). Finally, there was a significant difference between ML and HA cases in BA17 only ($p = 0.04$), with ML cases having significantly higher neurogranin levels than HA cases in this region. There were no detectable differences between LCR and LCD cases in neurogranin across all regions highlighting our immunohistochemistry labelling methods may not be as sensitive as western blotting.

4. Discussion

In the current study, we used novel methods to quantify differences in Ng levels in post-mortem brain tissue between Alzheimer's cases, healthy ageing participants without dementia, and mid-life controls. Further, we examined whether post-mortem Ng levels are associated with differences in lifetime cognitive ageing. We identified a reduction in relative Ng protein levels using across AD, HA, and ML cases.

Specifically, we report a significant decrease of Ng in AD cases relative to mid-life controls and healthy agers in total homogenate and synaptoneurosome preparations of BA20/21. Including enriched synaptoneurosome isolates in this study containing all the remaining synapses verified Ng decreases were not simply a result of neuronal loss. Immunohistochemical results supported this decline of Ng between ML, HA and AD where Ng levels were shown to be significantly lower in AD compared to healthy agers in BA17. Finally, in synaptoneurosome preparations of BA20/21, we report a significant decrease in Ng in healthy agers with poorer lifetime cognitive ageing relative to those with pre-morbid cognitive resilience.

This is the first known study to report a reduction of Ng in the temporal cortex (BA20/21) and the primary visual cortex (BA17) in AD, and is supported by prior reports of a significant decrease in Ng in the frontal cortex (Davidsson and Blennow, 1998), parietal cortex (Kvartsberg et al., 2019; Reddy et al., 2005b) and hippocampus (Davidsson and Blennow, 1998). In rodents, Ng protein expression and mRNA expression have also been reported to decrease with age (Mons et al., 2001). Within the healthy-ageing group, those with lifetime cognitive decline had lower Ng in synapses than those with lifetime cognitive resilience. This is the first known study to report differences in Ng by pre-morbid cognitive functions. Additionally, within the LCR sub-group higher levels of education were evident across cases displaying higher Ng levels suggestive environmental factors such as education may be associated with better cognition during ageing and may play a role in resilience.

Reduced Ng at the synapse could translate to age-related cognitive decline through altered LTP. Long-term potentiation is thought to be critical for the formation of long-term memories and requires the activation of N-methyl-D-aspartate (NMDA) receptors and an increase in post-synaptic Ca^{2+} (Bliss and Collingridge, 1993). This increase in Ca^{2+} activates Ca^{2+} /CaM-dependent protein kinase II (CaMKII), leading to the induction of LTP (Lledo et al., 1995; Zhong et al., 2009). As Ng can bind to CaM and influence its availability and distribution, it is plausible that reduced Ng in the ageing brain may lead to altered synaptic functioning, which could translate to age-related cognitive decline. There is evidence of both disrupted Ca^{2+} homeostasis (Nikolietopoulou and Tavernarakis, 2012) and a decrease in CaMKII activity (Davis et al., 2000) in the aged rodent brains and neurons.

The current study is the first known study to examine Ng across AD, healthy ageing, and young controls using synaptoneurosome preparations to examine levels within synaptic compartments, and IHC to provide a regional resolution of Ng expression across brain areas in human post-mortem tissue. The main limitations of this paper are the relatively small sample size of cases with cognitive data. Further, cognitive data were not available for AD and ML cases meaning inferences on the association between Ng and cognition in all samples was not possible. Where post-mortem data are a precious resource, especially with cognitive data spanning most of the life course, we acknowledge that a larger sample size would enable more reliable detection and estimation of effect sizes. Further research would benefit from larger sample sizes with pre-morbid cognitive data to examine any associations between brain Ng and cognitive decline. While we examined relative Ng levels in synaptic compartments, future work could also benefit from higher-resolution techniques, such as array tomography, to image Ng at the individual-synapse level. All techniques employed in this study used C terminus Ng antibodies. Whilst this is sufficient to determine Ng levels in brain tissue, combining future studies with a N terminus antibody may shed more light on degradation processes occurring whether it be full length or fragmented protein causing Ng to increase in CSF. Overall, we report a significant decrease in Ng in AD and within the healthy-ageing cohort associating with poorer lifetime cognition. Using synaptoneurosome preparations and IHC, we were able to examine differences at both a regional level and at the level of synaptic compartments. Our findings indicate that the increased Ng previously observed in the CSF of AD and ageing subjects reflects loss of Ng from the brain including loss of the protein in remaining synapses.

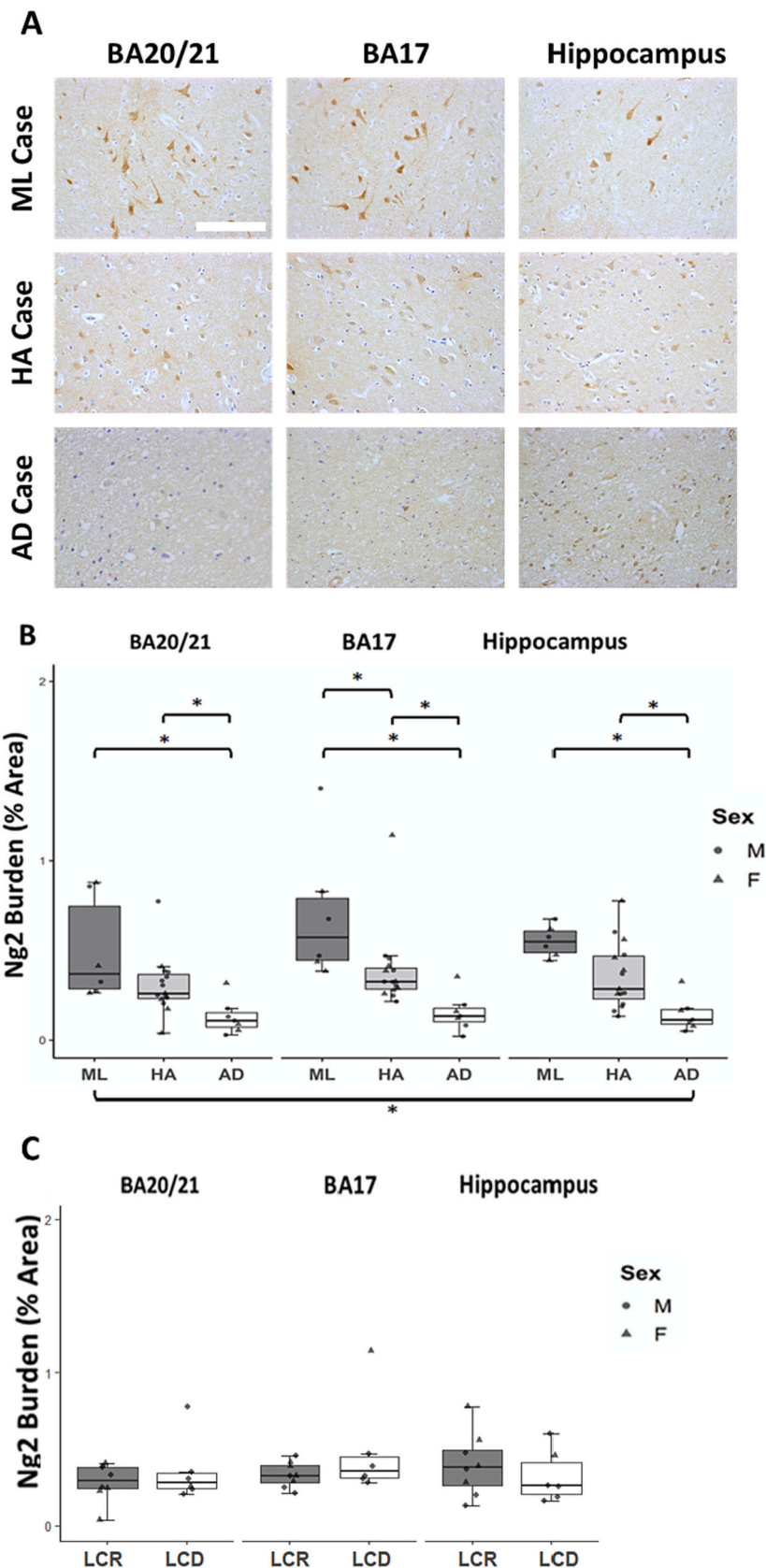


Fig. 2. Neurogranin protein levels decrease from mid-life through ageing and Alzheimer's Disease. **A** Representative IHC images from ML, HA and AD immunostained cases across three brain regions BA20/21, BA17 and hippocampus. **B** Ng2 measurements plotted across three brain regions show a step-wise decrease in neurogranin levels between cohorts. **C** Ng2 levels within the HA cohort split in relation to cognitive status as either lifetime cognitive resilient (LCR) or lifetime cognitive decline (LCD). For box-plots each case point represents case medians. * denotes $p < 0.05$ based on statistical analysis with linear mixed effects model (LMM) followed by ANOVA and post-hoc comparisons, Ng burden ~C; Cohort * Region + APOE + Sex + PMI + (1|Case/sample), followed by ANOVA. Scale bar 150 μ m for IHC images.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2023.105991>.

Declaration of Competing Interest

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

Data availability

Data will be made available on request.

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