Molecular Psychiatry (2021) 26:6023–6037
https://doi.org/10.1038/s41380-020-0797-2

ARTICLE

The human brain NGF metabolic pathway is impaired in the pre-clinical and clinical continuum of Alzheimers disease

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Received: 26 August 2019 / Revised: 12 May 2020 / Accepted: 19 May 2020 / Published online: 2 June 2020

Abstract

The NGF metabolic pathway entails the proteins that mature pro-nerve growth factor (proNGF) to NGF and those that degrade NGF. Basal forebrain cholinergic neurons require NGF for maintenance of cholinergic phenotype, are critical for cognition, and degenerate early in Alzheimer's disease (AD). In AD, NGF metabolism is altered, but it is not known whether this is an early phenomenon, nor how it relates to AD pathology and symptomology. We acquired dorsolateral/medial prefrontal cortex samples from individuals with Alzheimer's dementia, Mild Cognitive Impairment (MCI), or no cognitive impairment with high (HA-NCI) and low (LA-NCI) brain Aβ from the Religious Orders Study. Cortical proNGF protein, but not mRNA, was higher in AD, MCI, and HA-NCI, while mature NGF was lower. Plasminogen protein was higher in MCI and AD brain tissue, with plasminogen mRNA not likewise elevated, suggesting diminished activation of the proNGF convertase, plasmin. The plasminogen activator tPA was lower in HA-NCI while neuroserpin, the CNS tPA inhibitor, was higher in AD and MCI cortical samples. Matrix metalloproteinase 9 (MMP9), which degrades NGF, was overactive in MCI and AD. Transcription of the MMP9 inhibitor TIMP1 was lower in HA-NCI. ProNGF levels correlated with plasminogen, neuroserpin, and VAChT while NGF correlated with MMP9 activity. In NCI, proNGF correlated with cerebral Aβ and tau deposition and to cognitive performance. In summary, proNGF maturation is impaired in preclinical and clinical AD while mature NGF degradation is enhanced. These differences correlate with cognition, pathology, and cholinergic tone, and may suggest novel biomarkers and therapeutic targets.

Introduction

Alzheimer's disease (AD) pathomechanistic alterations commence 10–20 years before cognitive decline manifests [1, 2]. As a likely irreversible loss of functional cells and synapses underlies disease symptoms [3, 4], there is a need for new therapeutics targeting key AD pathophysiological mechanisms at the earliest possible stage. As such, a comprehensive understanding of early AD pathophysiology will be essential for the development of disease-modifying therapies [5].

Acetylcholinesterase inhibitors offer transient relief from cognitive decline [6] by potentiating the activity of acetylcholine at cortical and hippocampal cholinergic synapses, thereby partially compensating for a loss of cholinergic tone [7–10]. Indeed, basal forebrain cholinergic neurons (BFCNs) lose their phenotype and functionality early in the course of AD [10–12]. In early to moderate AD, the extent of the cholinergic deficit correlates well to the cognitive impairment [13, 14] and the efficacy of AChEIs correlates well to their pro-cognitive effects [15]. AChEIs improve cholinergic tone but do not prevent or slow the degeneration of the BFCN. A more sophisticated therapeutic capable of maintaining cholinergic function would likely provide greater relief for patients with Alzheimer's dementia.

Supplementary information The online version of this article (https://doi.org/10.1038/s41380-020-0797-2) contains supplementary material, which is available to authorized users.

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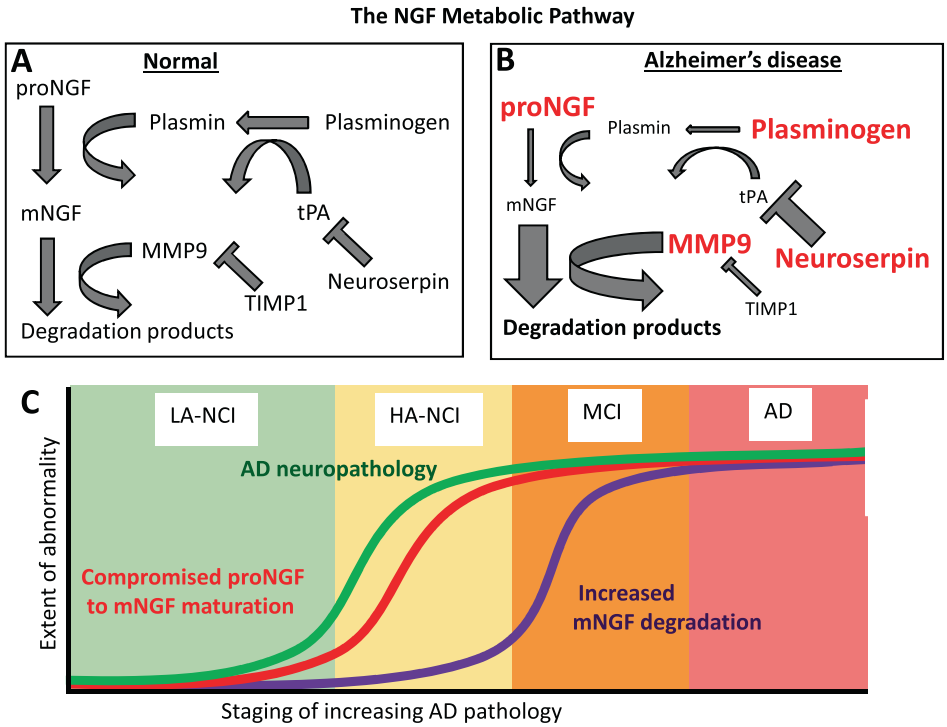


Fig. 1 Schematic representation of the NGF metabolic pathway and its compromise in the continuum of Alzheimer's disease. **a** ProNGF secreted from BFCN target cells is converted extracellularly to mature NGF (mNGF) by plasmin, which is derived from plasminogen by tissue plasminogen activator (tPA). The latter is regulated by its endogenous inhibitor, neuroserpin. Mature NGF is degraded by matrix metallo-protease 9 (MMP9), which is regulated by its endogenous inhibitor TIMP1 (tissue inhibitor of metallo-proteinases 1). **b** In Alzheimer's disease, neuroserpin levels are increased. The resulting deficit in tPA activity leads to impaired maturation of plasminogen to plasmin, evidenced by accumulating plasminogen. ProNGF likewise accumulates as it fails to be converted to mature NGF, which is decreased. Simultaneously, MMP9 activity is increased while its inhibitor, TIMP1, is decreased. **c** While deficits in proNGF maturation are evident alongside AD-like Aβ accumulation in individuals with no cognitive impairment (NCI), excessive NGF degradation is only evident in individuals diagnosed as MCI or AD.

However, the development of such a drug requires an understanding of the causes of cholinergic degeneration.

BFCN neurons, uniquely in the CNS, depend exclusively on the retrograde supply of nerve growth factor (NGF) for the maintenance of their (cholinergic) phenotype [16–19]. In AD, however, NGF transcription is normal [20] and the NGF precursor, proNGF, which lacks the same trophic function of mature NGF [21–24], is elevated [25].

Insight into this apparent paradox was provided by an ex vivo study in the rat cortex demonstrating that proNGF, and not mature NGF, was secreted in an activity-dependent manner alongside a set of proteins (the NGF metabolic pathway) responsible for converting proNGF to mature NGF and subsequently degrading mature NGF [26]. Briefly, proNGF is converted to NGF by plasmin, which is derived from the inactive zymogen, plasminogen, by tissue plasminogen activator (tPA). The activity of tPA is regulated by its central inhibitor, neuroserpin [27, 28]. Mature NGF is degraded by matrix metallo-protease 9 (MMP9), which is inhibited by TIMP1 [29, 30]. This pathway (Fig. 1a) has been shown to regulate the phenotype of cortical cholinergic synapses [31] and basal forebrain cholinergic cell bodies [32].

The proNGF to mNGF conversion has been shown to be compromised in human brain material from individuals with AD as early as in Mild Cognitive Impairment (MCI) [33, 34] and tPA activity was shown to be reduced in the brains of AD patients [35]. A transgenic rat modeling the human amyloid pathology recapitulates such NGF metabolic deficits [36] at stages with intracellular and extracellular amyloid deposition in the absence of tau, suggesting that NGF dysmetabolism could occur prior to the onset of dementia. However, the NGF metabolic pathway has not been fully assessed across the continuum of human AD pathology.

In this study, we investigated the protein and transcript levels of key players in NGF metabolism in the brains of individuals with Alzheimer's dementia, MCI, and cognitively normal individuals with (and without) AD-like levels of Aβ pathology. We also investigated the relationship between NGF metabolic markers and AD neuropathology at preclinical stages. We assessed the relationship between NGF metabolic markers and cholinergic tone, as assessed by VAChT staining. Finally, we assessed the associations of NGF metabolic pathway proteins to cognitive scores in

Table 1 Demographic and neuropathological characteristics of ROS brain donors included in this study.

	LA-NCI	HA-NCI	MCI	AD	*p* value (Chi square or ANOVA)
n =	43	16	19	20	
Percent female	53%	48%	58%	55%	0.82 (Chi-square test)
Age (years)	78.2 ± 7.5	84.5 ± 7.9	83.9 ± 7.6	82.7 ± 8.2	0.01 (ANOVA)
Post-mortem interval (hours)	10.4 ± 8.3	8.5 ± 10.0	8.9 ± 5.6	5.7 ± 3.7	0.16 (ANOVA)
Years of education	17.8 ± 3.9	18.3 ± 2.8	18.2 ± 4.0	18.1 ± 3.9	0.73 (ANOVA)
Cerebral amyloid-β percent area	0.4 ± 0.6	2.2 ± 2.1	2.8 ± 2.4	4.3 ± 3.5	<0.0001 (ANOVA)
NFT density/mm^2	1.6 ± 1.8	3.6 ± 4.8	5.1 ± 6.7	13.7 ± 19.1	<0.0001 (ANOVA)
CERAD	3.3 ± 1.1	2.3 ± 0.6	2.8 ± 1.1	1.9 ± 0.8	<0.0001 (ANOVA)
REAGAN	2.9 ± 0.5	2.3 ± 0.5	2.4 ± 0.7	2.2 ± 0.8	<0.0001 (ANOVA)
BRAAK	2.1 ± 1.2	3.7 ± 1.1	3.5 ± 0.8	3.6 ± 1.1	<0.0001 (ANOVA)
APOE ε4 allele frequency	0.12	0.20	0.18	0.17	0.31 (ANOVA)
Global Cognitive Score (z score)	0.18 ± 0.33	−0.04 ± 0.28	−0.22 ± 0.30	−2.44 ± 1.11	<0.0001 (ANOVA)

Data are presented as mean ± standard deviation. The *p* value column gives the result of a one-way ANOVA comparing the means of all four pathological groups or a X^2 test assessing their distribution.

LA-NCI low amyloid-no cognitive impairment, *HA-NCI* high amyloid-no cognitive impairment, *MCI* mild cognitive impairment, *AD* Alzheimer's disease, *NFT* neurofibrillary tangles.

cognitively normal individuals. We demonstrate that proNGF maturation is reduced in preclinical and clinical AD while mature NGF degradation is enhanced. These changes correlate with preclinical cognition and AD neuropathology, and may provide a platform for novel biomarkers and therapeutics.

Methods

Demographic characteristics of the study sample cohort

The Religious Orders Study (ROS) is a cohort of American clergy that undergo yearly cognitive evaluations, annual clinical assessments, and *post-mortem* brain donation [37]. Cognitive examinations include 21 tests of episodic memory, semantic memory, working memory, perceptual orientation, and processing speed which can be summarized as a Global Cognitive Score (GCS). *Post-mortem* data follow a uniform structured evaluation and provide measures of Aβ load (percent area of 6E10 Aβ immunoreactivity in eight regions: the CA1 and subiculum, the angular gyrus, the entorhinal cortex, the superior frontal cortex, the dorsolateral prefrontal cortex, the inferior temporal cortex, the anterior cingulate cortex, and the calcarine cortex) and tangle pathology (provided by stereological assessment of AT8 immunoreactivity across the entorhinal cortex, CA1, superior frontal cortex, mid frontal cortex, inferior temporal cortex, angular gyrus, cingulate gyrus, and calcarine cortex) [38]. Staging according to Braak, Reagan, and CERAD classifications were accomplished based on the nature and quantity of neuritic plaques and neurofibrillary tangles visualized with modified Bielschowsky silver staining.

We obtained 98 prefrontal cortex samples comprising 59 individuals with no cognitive impairment (NCI), 19 individuals diagnosed with MCI, and 20 individuals with clinically diagnosed Alzheimer's dementia. Samples were taken from Brodmann's areas 9 and 46, comprising the dorsolateral prefrontal cortex and part of the medial prefrontal cortex, areas implicated in the cholinergic modulation of cognition and in Alzheimer's-associated cognitive decline. Diagnoses of Alzheimer's dementia were made using the clinical criteria for AD recommended by the National Institute of Neurologic and Communicative Disorders and Stroke/AD and Related Disorders Association (NINCDS/ADRDA) [39]. Patients were diagnosed with MCI if determined by a blinded neuropsychologist to have a cognitive impairment while deemed by a clinician to not meet criteria for dementia. The demographic characteristics of the study groups are illustrated in Table 1.

Classification of preclinical Aβ pathology

To determine the relationship between Aß-amyloidosis and NGF dysmetabolism in AD-asymptomatic Aß-positive individuals, we established a cut-off point equivalent to two standard errors below the mean Aβ scores of the MCI group

Table 2 Western blotting parameters.

Antibody (target)	Clonality	Source	Concentration	SDS-PAGE Gel %	Molecular weight
Ab9795 (NGF)	Polyclonal	Abcam (rabbit)	1:1000	12%	13 kDa
ANT-005 (proNGF)	Polyclonal	Alomone labs (rabbit)	1:2500	10%	27 kDa; 41 kDa
Ab154560 (plasminogen)	Polyclonal	Abcam (rabbit)	1:5000	8%	88 kDa
Anti-neuroserpin	Polyclonal	Dr. Daniel Lawrence, U. Michigan (rabbit)	1:10,000	10%	47 kDa
MAB374 (GAPDH)	Monoclonal	Millipore (mouse)	1:10,000	8–12%	37 kDa
139103 (VAChT)	Polyclonal	SYSY (Rabbit)	1:2000	10%	57 kDa; 75 kDa

Table 3 Primers for qPCR analysis of NGF metabolic pathway markers.

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
NGF	CATCATCCCATCCCATCTTC	GTCTGTGGCGGTGGTCTTAT
PLASMINOGEN	GCCCCATAGACACAGCATTT	TAGCACCAGGGACCACCTAC
NEUROSERPIN	GTAGCCGTGGCCAACTACAT	CCCTTGGGGATACCAAATCT
TPA	GACGTGGGAGTACTGTGATGTG	CCCTCCTTTGATGCGAAACTGA
MMP-9	GCCATTCACGTCGTCCTTAT	TTGACAGCGACAAGAAGTGG
TIMP-1	TGACATCCGGTTCGTCTACA	TGCAGTTTTCCAGCAATGAG
HPRT	TTGCTTTCCTTGGTCAGGCA	ATCCAACACTTCGTGGGGTC
ACTIN	ACAGCCTGGATAGCAACG	CACCAACTGGGACGACAT
GAPDH	CCCCACTTGATTTTGGAGGGA	AGGGCTGCTTTTAACTCTGGT

which divided NCI into low-Aβ expressing (LA-NCI; $n = 43$) and high-Aβ expressing (HA-NCI; $n = 16$) groups, with the latter representing an AD/MCI-like Aβ.

Analysis of the NGF metabolic pathway by qPCR, Western Blotting, ELISA, and gelatin zymography

Protein and transcript levels of NGF metabolic pathway proteins and the cholinergic synapse marker VAChT were assays by qPCR, Western Blotting, and ELISA using established techniques [26, 34], as detailed in the Supplementary Methods. Western Blotting parameters are outlined in Table 2, while qPCR primers are listed in Table 3. Representative blots with positive controls are shown in Supplementary Fig. 1 following the parameters outlined in Table 2; briefly, the Alomone ANT-005 antibody was used to reveal 27 kDa (unmodified) and 41 kDa (glycosylated) proNGF species (Supplementary Fig. 1a), as we have previously reported [40], while mature NGF immunoreactivity was revealed at 14 kDa with the abcam NGF 9795 antibody subsequent to a chloroform/methanol protein extraction (Supplementary Fig. 1b; following the protocol of Locke et al. [41]; see Supplementary Methods); this extraction is necessary to detect NGF at the low abundance at which it exists in the human cortex and while the antibody reacts with a proNGF control, higher molecular weight bands are faint compared with the 14 kDa band (Supplementary Fig. 1b). Plasminogen and neuroserpin were revealed with ab154560 (abcam, Cambridge, UK) and an anti-neuroserpin provided by Dr Daniel Lawrence (Supplementary Fig. 1c and d). MMP9 and proMMP9 activity was assayed using gelatin zymography following [33, 40].

Statistical analysis

Group statistics were performed using GraphPad Prism 5 (GRAPHPAD Software, San Diego, California, USA). One-way ANOVAs with Bonferroni post-hoc tests or Kruskal–Wallis tests with Dunn's post-hoc corrections were used to compare group means, depending on the adherence of each sample to a normal distribution as assessed by a D'Agostino-Pearson Omnibus Normality Test. Correlations were examined in R using multiple linear regression, with age and sex included as cofactors and partial r^2 and p values reported for the species of interest; plots were prepared with GRAPHPAD. All values were normalized to the mean of the LA-NCI group and represented as a fold-change from that mean. Differences in demographic characteristics were examined using ANOVA or chi-square tests as appropriate (see Table 1). The effects of age and sex on each protein or transcript were examined using linear regression and unpaired Student's t-tests (see Supplementary Table 1).

Results

Demographic characteristics of the study cohort (see Table 1)

No differences in sex proportions, years of education, *post-mortem* interval, or APOE ε4 allele frequency were observed between groups. The low- Aβ NCI group was younger than the high- Aβ NCI group. As expected, the HA-NCI, MCI, and AD groups had higher Aβ deposition than the LA-NCI group; this area was greater in AD compared with HA-NCI and MCI, though there was no difference between the latter groups. The AD groups exhibited more cortical tangles than the LA-NCI and HA-NCI; no other differences were observed. Average CERAD neuropathological diagnostic scores (definite, probable, possible, and no AD; coded 1–4) [42] were lower in HA-NCI, MCI, and AD compared with LA-NCI. Average Reagan diagnoses (low, intermediate, and high likelihood of AD, coded 1–4), were higher in HA-NCI and AD compared to the LA-NCI group; no other differences were observed. Similarly, average Braak stage was higher in HA-NCI, MCI, and AD versus LA-NCI. Global Cognitive Scores were lower in AD compared with each of LA-NCI, HA-NCI, and MCI; no other differences were observed.

Effects of age and sex

Weak correlations were observed between MMP9 activity and age and proNGF 41 kDa protein and age. All other associations were not significant and no effects of sex were observed (see Supplementary Table 1).

Protein and mRNA expression of NGF across the AD continuum

We investigated both mature NGF and proNGF protein levels, as well as the associated NGF transcript, to assess the likely availability of trophic support to BFCN neurons in the continuum of AD pathology. We assessed both 27 kDa and 41 kDa proNGF variants, with the former representing unprocessed proNGF and the latter representing the secreted form [26].

While we observed no difference in the expression of the NGF transcript between the different clinical groups (Fig. 2a; Table 4), the mature NGF protein was lower in dorsolateral/medial prefrontal cortex homogenates from MCI and AD cases compared with LA-NCI, with a trend towards a reduction in the HA-NCI group (Fig. 2b; Table 4). Both 27 kDa and 41 kDa proNGF protein immunoreactivity in individuals classified as HA-NCI, MCI, or AD as compared with those classified as LA-NCI were higher (Fig. 2c, d; Table 4).

Investigation of the plasmin activating system across the AD continuum

To determine the impact of AD pathology on the conversion of proNGF to mature NGF and possible underlying mechanisms, we investigated the protein and transcript levels of plasminogen, tPA, and neuroserpin.

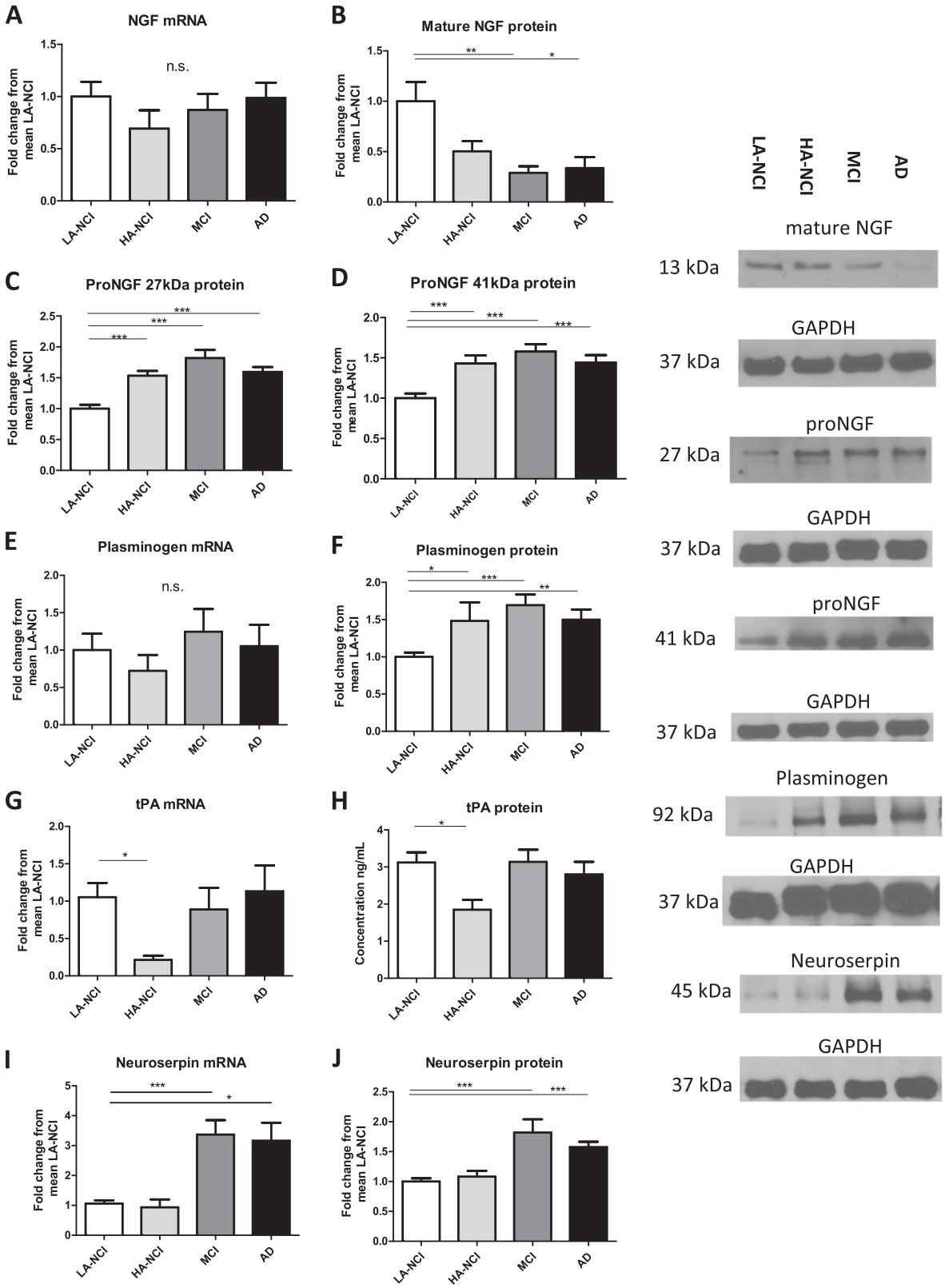
Although plasminogen mRNA levels were not significantly different between groups (Fig. 2e; Table 4), plasminogen protein expression was higher in dorsolateral/medial prefrontal cortex homogenates from HA-NCI, AD, and MCI individuals compared with LA-NCI (Fig. 2f; Table 4). The expression of tissue plasminogen activator (tPA) was lower in HA-NCI compared with LA-NCI, but MCI and AD levels were similar to LA-NCI, both as a transcript (Fig. 2g) and as protein (Fig. 2h; Table 4). However, the mRNA levels of the tPA inhibitor, neuroserpin, were higher in MCI and AD (Fig. 2i; Table 4), which was mirrored by the expression of neuroserpin protein (Fig. 2j; Table 4).

Protein and mRNA expression of MMP9 and TIMP1 across the AD continuum

AD and MCI individuals showed increased mRNA levels of MMP9, a prominent NGF-degrading protease [26], though there was no difference between low and high Aβ NCI individuals (Fig. 3a; Table 4). Dorsolateral/medial prefrontal cortex homogenates from such cases also had higher MMP9 proteolytic activity as measured by gelatin zymography at MCI and AD clinical stages (Fig. 3b; Table 4). At the same stages, the activity of proMMP9 was correspondingly increased (Fig. 3c; Table 4). Consistently with the above, transcript levels of the MMP9 inhibitor TIMP1 were significantly lower in MCI and AD but not in HA-NCI (Fig. 3d; Table 4).

Associations between the NGF metabolic pathway proteins and proNGF

NGF dysmetabolism would predict higher proNGF levels with higher plasminogen and neuroserpin, reflecting a diminished proNGF conversion to mature NGF, causing an accumulation of unmatured proNGF. Indeed, levels of the 27 kDa proNGF protein were positively correlated with levels of plasminogen (Supplementary Fig. 2a; Supplementary Table 2) and to neuroserpin (Supplementary Fig. 2b; Supplementary Table 2) in the whole sample, as were levels of 41 kDa proNGF (Supplementary Fig. 2c; Supplementary Table 2). Levels of neuroserpin, the endogenous tPA inhibitor, also correlated with levels of plasminogen (Supplementary Fig. 2e; Supplementary Table 2). TIMP1 mRNA correlated negatively to MMP9 activity


A
NGF mRNA
n.s.
Fold change from mean LA-NCI
LA-NCI
HA-NCI
MCI
AD
B
Mature NGF protein
C
ProNGF 27kDa protein
D
ProNGF 41kDa protein
E
Plasminogen mRNA
F
Plasminogen protein
G
tPA mRNA
H
tPA protein
Concentration ng/mL
I
Neuroserpin mRNA
J
Neuroserpin protein
LA-NCI
HA-NCI
MCI
AD
mature NGF
13 kDa
GAPDH
37 kDa
proNGF
27 kDa
GAPDH
37 kDa
proNGF
41 kDa
GAPDH
37 kDa
Plasminogen
92 kDa
GAPDH
37 kDa
Neuroserpin
45 kDa
GAPDH
37 kDa

◀ **Fig. 2 Normal NGF synthesis in the continuum of AD pathology is accompanied by an increase of proNGF and decrease of mNGF, beginning at preclinical stages, as well as abnormal expression of proteins participating in proNGF maturation. a** No differences in NGF mRNA were observed by qPCR. **b** Decreased mNGF immunoreactivity at 13 kDa in MCI/AD brains. **c** Increased proNGF immunoreactivity at 27 kDa in HA-NCI/MCI/AD brains. **d** Increased proNGF immunoreactivity at 41 kDa in HA-NCI/MCI/AD brains. **e** Dorsolateral/medial prefrontal cortex plasminogen mRNA does not differ between LA-NCI, HA-NCI, MCI, and AD. **f** Plasminogen protein is elevated in dorsolateral/medial prefrontal cortex homogenates from HA-NCI, MCI, and AD individuals versus those from LA-NCI individuals. **g** tPA mRNA in dorsolateral/medial prefrontal cortex homogenates is decreased in HA-NCI and unchanged in MCI and AD versus LA-NCI. **h** tPA protein is likewise solely decreased in HA-NCI vs. LA-NCI. **i** Neuroserpin mRNA is increased in dorsolateral/medial prefrontal cortex homogenates in AD and MCI versus LA-NCI. **j** Levels of neuroserpin protein are higher in dorsolateral/medial prefrontal cortex homogenates from individuals diagnosed with MCI/AD. Representative Western blots are shown for NGF at 13 kDa, proNGF at 27 and 41 kDa, plasminogen at 92 kDa, and neuroserpin at 45 kDa with 37 kDa GAPDH as the reference protein. Groups were LA-NCI; $n = 43$, HA-NCI; $n = 16$, MCI; $n = 20$, or AD; $n = 19$. All comparisons performed with a one-way ANOVA and Bonferroni post-hoc tests or a Kruskal-Wallace test and Dunn's post-hoc tests. All bars indicate mean + SEM.

(Supplementary Fig. 2f; Supplementary Table 2). As predicted, levels of mature NGF (but not proNGF) correlated with MMP9 activity (Supplementary Fig. 2g; Supplementary Table 2).

Associations between NGF pathway markers and neuropathological measures in individuals with no cognitive impairment (NCI)

To investigate the relationship between preclinical AD pathology and NGF metabolism, we correlated quantitative measures of Aβ and tau pathology to proNGF protein levels in individuals with NCI.

The 27 kDa variant of proNGF positively correlated with cerebral Aβ deposition (Fig. 4a; Table 5). This form of proNGF also correlated with a score of cortical tangle pathology (Fig. 4b; Table 5). The 41 kDa variant of proNGF also correlated with cortical Aβ deposition (Fig. 4c; Table 5) and tangle burden (Fig. 4d; Table 5). Furthermore, proNGF 41 kDa protein levels were differentially expressed according to Reagan diagnosis in NCI individuals, with brains from individuals classified as Reagan 3 or 4 expressing more proNGF compared with that of those classified as 2, and 4 having higher proNGF than 3 (Fig. 4e; Table 5). When NCI cases were separated according to the Braak index of tauopathy, higher proNGF 41 kDa protein was found in individuals classified as Braak stage 4–5 than those classified as Braak 0–1 (Fig. 4f; Table 5). No correlations to or differential expression of other NGF pathway markers by these variables were observed.

Associations between NGF pathway markers and pre-mortem cognitive scores in NCI

To understand the consequences of NGF dysmetabolism prior to the onset of symptomatic AD, we correlated protein levels of NGF pathway markers in individuals with NCI to cognitive scores, given by the overall *z*-scores of the last Global Cognitive Score (CGS) assessment prior to death. GCS *z*-scores correlated negatively to the 27 kDa band immunoreactive for proNGF (Fig. 4g; Table 5) and to the 41 kDa proNGF variant (Fig. 4h; Table 5). We also observed a positive correlation between tPA protein levels and GCS *z*-scores (Fig. 4i; Table 5) in patients without overt cognitive impairment. No other association with changes in cognitive scores was observed in the NCI group.

Investigation of VAChT across the AD continuum and its association with proNGF accumulation

To validate the relevance of NGF dysmetabolism to the cortical cholinergic phenotype, we correlated protein levels of the cholinergic synapse marker VAChT with proNGF protein levels in a subset ($n = 84$) of our samples. The 57 kDa unprocessed form of VAChT was significantly reduced in MCI and AD (Fig. 5a; Table 4), while the 75 kDa glycosylated isoform was reduced only in the AD group (Fig. 5b; Table 4). Importantly, 57 kDa VAChT protein levels correlated with both 27 kDa and 41 kDa proNGF (Fig. 5c and d; Supplementary Table 2), although the glycosylated form missed significance. Interestingly, we observed that the expression of neuroserpin was also significantly associated with both isoforms of VAChT (Fig. 5e and f; Supplementary Table 2).

Discussion

In this report we provide a thorough analysis of the NGF metabolic pathway across the continuum of AD pathology, including preclinical and clinical stages, in dorsolateral/medial prefrontal cortex samples from individuals with AD, MCI, and AD-asymptomatic individuals with AD-like Aβ deposition. We demonstrate higher levels of proNGF and plasminogen protein, in the absence of transcriptional differences in associated genes, suggesting impairments to the plasminogen activating system throughout HA-NCI, MCI, and AD. We confirmed this by demonstrating higher neuroserpin in MCI and AD and lower tPA in NCI individuals with higher brain Aβ deposition, which suggest decreased plasmin maturation resulting in impaired proNGF maturation to mNGF. Simultaneously, higher MMP9 occurs concurrently with lower TIMP1, resulting in lower NGF as evidenced by the reduced levels of mature NGF in MCI and AD (see Fig. 1b). Our results suggest that the conversion of

Table 4 Comparisons of NGF metabolic pathway proteins and VAChT in frontal cortex between individuals classified LA-NCI ($n = 43$), HA-NCI ($n = 16$), MCI ($n = 20$), and AD ($n = 19$).

Analyte	LA-NCI vs HA-NCI	LA-NCI vs. MCI	LA-NCI vs AD	Overall
NGF mRNA	n.s.	n.s.	n.s.	df = 3.95; $H = 2.4$; $p > 0.05$
NGF protein	rs = 8.842, $p = 0.06$	rs = 15.73, $p < 0.01$	rs = 17.14, $p < 0.01$	df = 3.81; $H = 14.38$; $p < 0.01$
proNGF 27 kDa	$t = 4.095$, $p < 0.001$	$t = 7.058$, $p < 0.001$	$t = 4.984$ $p < 0.001$	df = 3.95; $F = 19.96$; $p < 0.0001$
proNGF 41 kDa	$t = 3.795$, $p < 0.001$	$t = 5.399$, $p < 0.001$	$t = 4.035$, $p < 0.001$	df = 3.95; $F = 12.68$; $p < 0.0001$
Plasminogen mRNA	n.s	n.s	n.s	df = 3.95; $H = 0.87$; $p > 0.05$
Plasminogen protein	$t = 2.605$, $p < 0.05$	$t = 4.035$, $p < 0.001$	$t = 3.070$ $p < 0.01$	df = 3.95; $F = 26.80$; $p < 0.0001$
tPA mRNA	rs = 22.37, $p < 0.05$	n.s	n.s	df = 3.95; $H = 7.652$; $p = 0.05$
tPA protein	rs = 23.46, $p < 0.05$	n.s	n.s	df = 3.95; $H = 8.754$, $p < 0.05$
Neuroserpin mRNA	n.s	rs = 30.91, $p < 0.001$	rs = 22.26, $p < 0.05$	df = 3.95; $H = 29.23$; $p < 0.0001$
Neuroserpin protein	n.s	rs = −34.41, $p < 0.001$	rs = -33.39 $p < 0.001$	df = 3.95; $H = 30.18$; $p < 0.0001$
MMP9 mRNA	n.s	rs = 26.67, $p < 0.001$	rs = 28.63, $p < 0.001$	df = 3.95; $H = 25.44$; $p < 0.0001$
MMP9 activity	n.s	rs = 27.55, $p < 0.001$	rs = 33.91 $p < 0.001$	df = 3.95; $H = 29.33$; $p < 0.0001$
proMMP9 activity	n.s	rs = 27.55, $p < 0.001$	$t = 33.91$ $p < 0.001$	df = 3.95; $H = 29.33$; $p < 0.0001$
TIMP1 mRNA	n.s	rs = 18.90, $p < 0.05$	rs = 26.40, $p < 0.01$	df = 3.95; $H = 15.03$; $p < 0.01$
VAChT 57 kDa	n.s	rs = 19.71, $p < 0.05$	rs = 24.12, $p < 0.01$	df = 3.81; $H = 16.24$; $p < 0.001$
VAChT 75 kDa	n.s	$t = 2.811$, $p < 0.05$	$t = 3.272$, $p < 0.01$	df = 3.81; $F = 5.86$; $p < 0.01$

Results of ANOVA/Kruskal-Wallis comparison and Bonferroni/Dunn's post-hoc tests are reported.

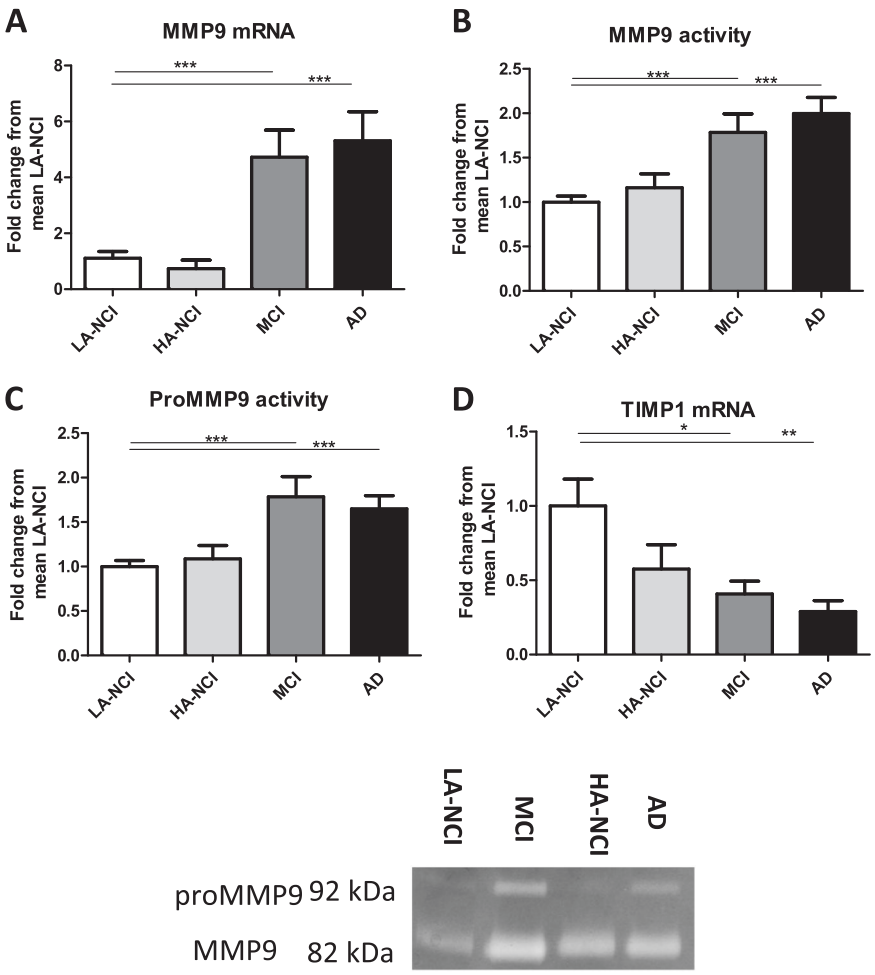


Fig. 3 Increased levels and protease activity of MMP9, a mature-NGF degrading protease, and diminished expression of its endogenous inhibitor, TIMP1, at AD clinical stages in dorsolateral/medial prefrontal cortex homogenates. a Increased expression of MMP9 mRNA in MCI and AD was observed by qPCR. **b** Gelatin zymography revealed increased MMP9 activity in MCI and AD individuals. **c** ProMMP9 activity was increased in MCI and AD. Representative zymograms are shown for MMP9 at 82 kDa and proMMP9 at 92 kDa. **d** Using qPCR, we observed less TIMP1 mRNA in individuals with MCI or AD. Refer to Fig. 1 legend for cases, groups, and statistics.

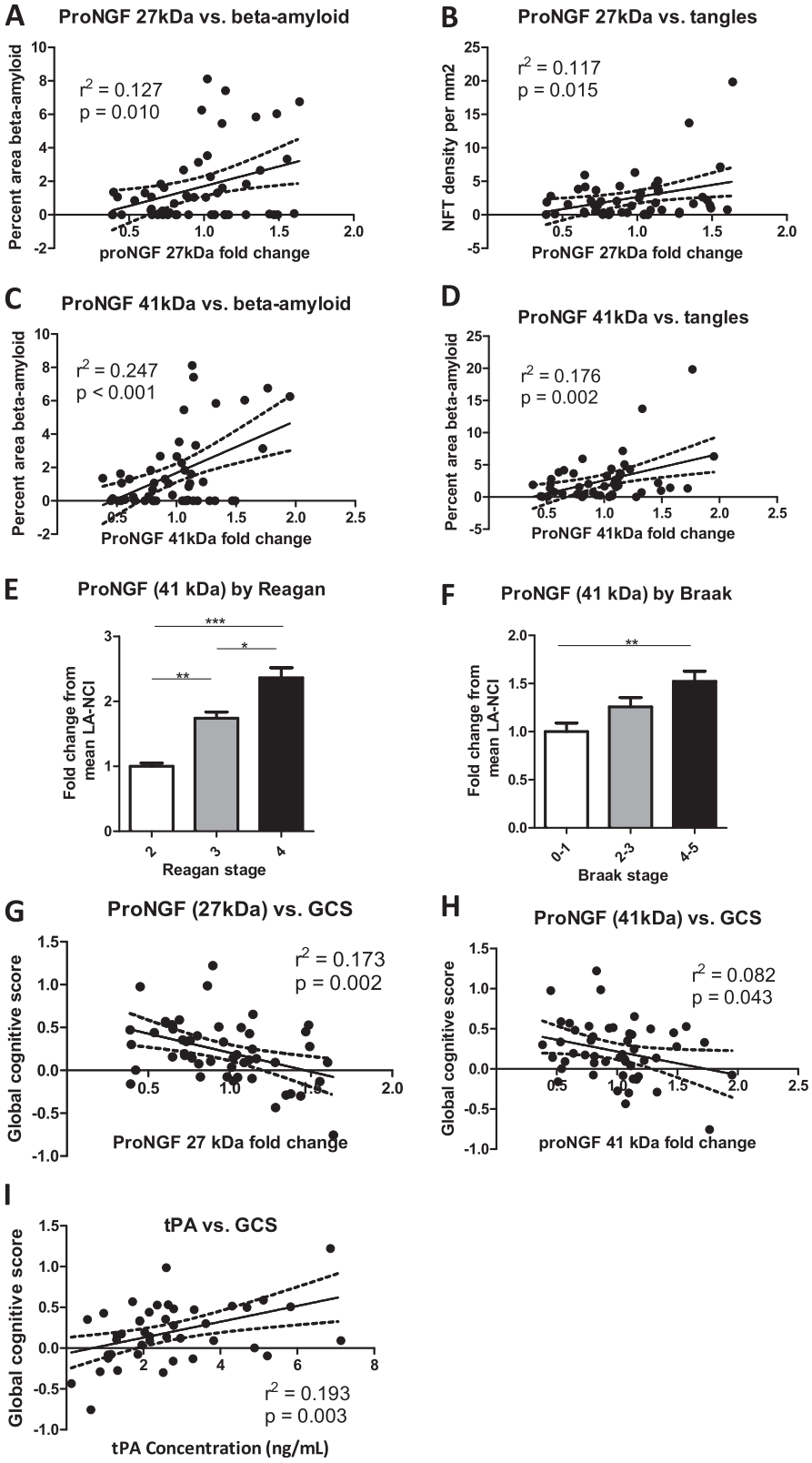


Fig. 4 Association between elevations of dorsolateral/ medial prefrontal cortex proNGF protein levels and hallmarks of Alzheimer's neuropathology and to cognitive scores in individuals with no cognitive impairment ($n = 59$). **a** The 27 kDa variant of proNGF correlates with amyloid deposition in aged individuals without cognitive impairment. **b** The 27 kDa variant of proNGF correlates with an index of tangle pathology. **c** ProNGF immunoreactivity at 41 kDa also correlates with amyloid deposition. **d** ProNGF 41 kDa protein correlates with tangle pathology. **e** ProNGF 41 kDa protein is differentially regulated by Reagan score, with higher levels in individuals classified as Reagan 2 ($n = 20$) and Reagan 3 ($n = 31$) than in Reagan 4 ($n = 8$), as well as in Reagan 2 compared with Reagan 3. **f** The 41 kDa isoform of proNGF was also differentially expressed by Braak stage, with higher levels in brains classified as Braak 4–5 ($n = 17$) compared with those classified as Braak 0–1 ($n = 27$), with the intermediate Braak 2–3 brains ($n = 15$) not significantly different from either. **g** ProNGF 27 kDa protein correlates negatively to Global Cognitive Score z-scores. **h** ProNGF immunoreactivity at 41 kDa correlates with Global Cognitive Score z-scores. **i** tPA protein concentrations measured by ELISA correlate with Global Cognitive Score z-scores. All comparisons performed with a one-way ANOVA and Bonferroni post-hoc tests or a Kruskal–Wallace test and Dunn's post-hoc test. Bars indicate mean + SEM. All relationships assessed by multiple linear regression with age and sex as covariates, with 95% confidence intervals and partial r^2 and p values displayed.

proNGF is impaired alongside Aβ pathology in preclinical AD, while excessive NGF degradation is a feature of clinically established AD and MCI (see Fig. 1c).

We validate the NGF metabolic pathway by demonstrating correlations between proNGF or mature NGF levels and the levels of relevant maturing or degrading enzymes,

Table 5 Associations between NGF metabolic pathway proteins and AD neuropathology, comparisons of NGF metabolic pathway proteins between Reagan and Braak classifications, and associations of NGF metabolic pathway markers with preclinical cognition in individuals classified as NCI ($n = 59$).

Protein	Correlate	Statistics (partial r^2 and p values for correlates in an age/sex corrected multiple regression model or results of one-way ANOVA)
27 kDa proNGF	Aβ deposition	$r^2 = 0.127$; $p = 0.01$
27 kDa proNGF	tangles	$r^2 = 0.117$; $p < 0.05$
41 kDa proNGF	Aβ deposition	$r^2 = 0.247$, $p < 0.001$
41 kDa proNGF	tangles	$r^2 = 0.176$, $p < 0.01$
41 kDa proNGF	Reagan stage	df = 2, 56; $F = 14.40$; $p = 0.007$; Reagan 2 vs Reagan 3, $t = 2.825$ $p < 0.051$; Reagan 2 vs Reagan 4, $t = 4.965$ $p < 0.001$; Reagan 3 vs Reagan 4, $t = 3.753$ $p < 0.05$
41 kDa proNGF	Braak stage	df = 2, 56; $F = 14.40$; $p = 0.007$; Reagan 2 vs Reagan 3, $t = 2.825$ $p < 0.051$; Reagan 2 vs Reagan 4, $t = 4.965$ $p < 0.001$; Reagan 3 vs Reagan 4, $t = 3.753$ $p < 0.05$
27 kDa proNGF	Global cognitive z-scores	$r^2 = 0.173$, $p < 0.01$
41 kDa proNGF	Global cognitive z-scores	$r^2 = 0.082$, $p < 0.05$
tPA protein	Global cognitive z-scores	$r^2 = 0.193$, $p < 0.01$

Partial r^2 and p values are reported for multiple regression models, with age and sex as cofactors.

supporting its role as a critical determinant of trophic support to BFCN. Most importantly, we illustrate the association of proNGF and tPA with cognitive function in AD-asymptomatic cognitively normal individuals, and we also show that reductions in VAChT staining associate with the accumulation of proNGF and neuroserpin. These results support the hypothesis that the AD-related atrophy of NGF-dependent BFCN is a consequence of the withdrawal of their trophic support, a concept demonstrated in vivo by the immunoneutralization of endogenous NGF or the blocking of TrkA receptors [19], as well as pharmacologically by the inhibition of plasmin activation and therefore the conversion of proNGF to mNGF [31, 32]. As such, alterations to the NGF metabolic pathway could serve as a platform for developing novel pro-cognitive cholinergic therapeutics or biomarkers of cognitive decline.

A link between amyloid-β pathology and NGF metabolism

Herewith we found consistent associations between levels of Aβ pathology and the various dysregulated markers of NGF metabolism, in particular proNGF, at preclinical disease stages. These results accord with our previous findings in individuals with Down syndrome, a condition characterized by lifelong amyloidosis [43, 44], in which we described a similar dysregulation of NGF metabolism in frontal cortex which correlated with Aβ pathology [40]. We have also previously shown that injected soluble Aβ oligomers *per se* can induce rapid proNGF accumulation and increased MMP9 activation in the brains of naïve rats [34] and that both rats and mice transgenic for human mutated APP display a lifelong deregulation of NGF metabolism in the absence of tau pathology [34, 36]. While we did observe certain correlations between tauopathy burden and markers of NGF dysmetabolism, these were less strong and consistent than those with Aβ. These studies therefore support NGF metabolic dysfunction as following primarily, though not necessarily exclusively, from Aβ accumulation in AD.

While this study did not investigate the direct mechanism by which Aβ disrupts NGF metabolism, previous results have demonstrated that the application of an anti-inflammatory is sufficient to resolve the NGF dysmetabolism induced by the injection of Aβ oligomers into the hippocampi of naïve rats [34]. Indeed, many proteins involved in the NGF metabolic pathway, such as the plasmin activating system and MMP9, have roles in pro-inflammatory pathways including cytokine activation, glial remodelling, and phagocytosis [45–47]. Aβ is known to induce strong and inflammatory reactions that become chronic in the context of progressive amyloid pathology [48, 49] and growing evidence suggests that early CNS inflammation plays a critical role in the pathogenesis of AD [50]. NGF dysmetabolism and cholinergic degeneration may therefore be a consequence of this prolonged inflammatory activation provoked by Aβ [51, 52]. Intriguingly, the cholinergic system has an established anti-inflammatory role that might permit it to attenuate the degenerative effects of chronic neuroinflammation in AD [53].

A revisited model explaining cholinergic degeneration in Alzheimer's disease

Several explanations for the preferential vulnerability of BFCN in AD have been proffered, including reductions in TrkA levels in the context of normal p75ntr expression [54], vulnerability to tauopathy [55], and impaired axonal transport [56, 57]. While further research is warranted, a

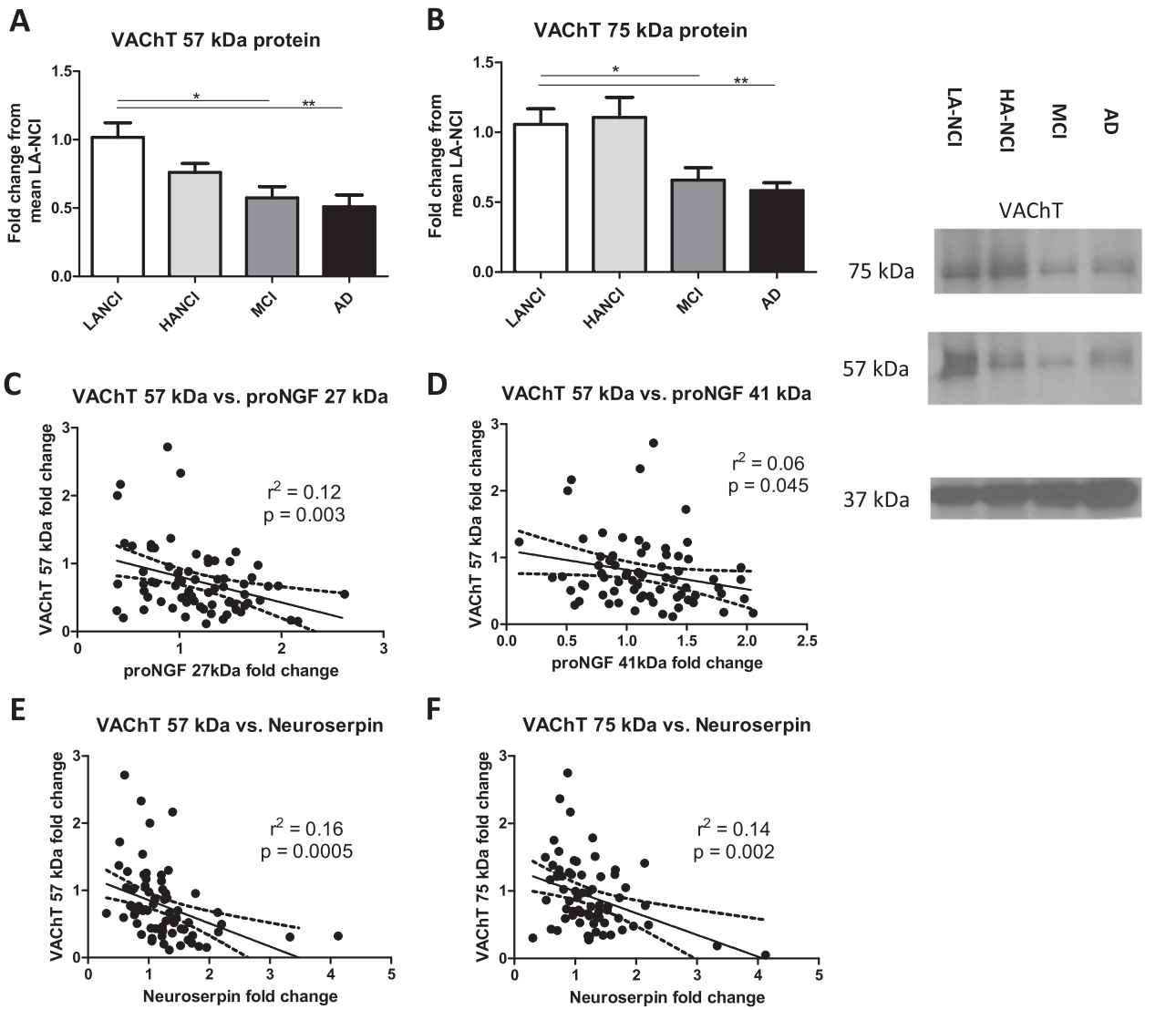


Fig. 5 VAChT expression across the AD continuum and its association with proNGF and neuroserpin expression. **a** VAChT immunoreactivity at 57 kDa is significantly reduced in MCI and in AD. **b** VAChT immunoreactivity at 75 kDa is significantly reduced in MCI and in AD. **c** VAChT immunoreactivity at 57 kDa significantly correlates with proNGF immunoreactivity at 27 kDa. **d** VAChT immunoreactivity at 57 kDa significantly correlates with proNGF immunoreactivity at 41 kDa. **e** VAChT immunoreactivity at 57 kDa significantly correlates with neuroserpin protein. **f** VAChT immunoreactivity at 75 kDa significantly correlates with neuroserpin protein. Representative Western blots are shown for VAChT at 75 kDa and 57 kDa, with 37 kDa GAPDH as the reference protein. Groups were LA-NCI; $n = 36$, HA-NCI; $n = 14$, MCI; $n = 17$, or AD; $n = 17$. All comparisons performed with a one-way ANOVA and Bonferroni post-hoc tests or a Kruskal–Wallace test and Dunn's post-hoc tests. All bars indicate mean + SEM. All relationships were assessed by multiple linear regression with age and sex as covariates, with 95% confidence intervals and partial r^2 and p values displayed.

dysfunctional NGF metabolism could lie at the root of the cholinergic pathology. The expression of the *NTRK1* (TrkA) gene is ultimately dependent on NGF signalling through its high-affinity receptor TrkA [58, 59], as is the cholinergic gene locus containing the choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) genes [60–65], and also genes critical for axonal integrity and transport [66, 67]. Therefore, the decline of TrkA levels in AD and the consequent predomination of p75/sortilin-mediated signalling could result from the compromise of NGF metabolism and the subsequent reduction of mature NGF available to the basal forebrain. Furthermore, the NGF metabolic pathway has been demonstrated to be responsible for the maintenance of the synaptic [31] and somato-dendritic [32] cholinergic phenotype of BFCNs in vivo.

The NGF metabolic pathway and biomarker studies

We have previously shown that proteins involved in NGF metabolism, measured in biofluids, may serve as biomarkers of AD pathology and AD-associated cognitive decline. Self-

to-self increases in proNGF levels measured in the plasma of AD-asymptomatic individuals with Down syndrome were effective predictors of cognitive decline across 2 years of follow-up [68]. Retrospective analysis of a cohort of plasma from the general population showed significant associations between various markers of NGF metabolism such as MMP9 and the risk of dementia onset [69]. Furthermore, CSF from patients with MCI and AD showed altered levels of metallo-proteases and the plasminogen activating system, both critical elements of the NGF metabolic pathway [70]. These results are in line with reports that CSF proNGF is increased in AD and that this increase correlates with disease staging and cognitive impairments [71]. This study indicates that such changes occur in step with similar stepwise changes in the brain (see Fig. 1b, c), with Aβ as the most likely mediator, beginning at the earliest stages of the disease. As new, reliable, and cost-effective biomarkers of incipient Alzheimer's pathology are a critical unmet need for the treatment of AD [72], it will be important to validate proNGF and related markers in other cohorts in this capacity. If positive, it will also be essential to develop reliable ELISA assays or PET tracers capable of distinguishing proNGF from mature NGF.

Relevance of NGF metabolism for cognition and therapeutics

The forebrain cholinergic system plays a vital role in cognition by inducing specific information-processing states in target tissues [73]. Previous studies have heavily implicated the cholinergic system in the cognitive deficit in early-to-moderate Alzheimer's dementia [74].

Our results support a role for cholinergic dysfunction in early cognitive decline in AD, demonstrating that correlations between proNGF processing and reduced performance on cognitive tests can be observed even in ostensibly healthy, cognitively unimpaired patients. Furthermore, we saw that VAChT immunoreactivity was reduced in the AD continuum, and that it correlated with increases in proNGF and neuroserpin immunoreactivity. VAChT levels were not reduced in the high-amyloid NCI group, suggesting that gross changes to proNGF levels precede changes to levels of cholinergic markers. Indeed, others have observed cholinergic markers to be maintained in the early phases of AD [75, 76], and we have proposed a model in which the disruption of the cholinergic signalling initially manifests as a re-organization or sprouting of terminals as BFCNs seek to re-establish their lost trophic support [77].

While AChEIs are only transiently efficacious, it is remarkable that cholinergic therapy can still exert pro-cognitive effects at stages with devastating brain damage already established. A more sophisticated therapy aimed at re-establishing a sustained cholinergic tone, perhaps by enhancing proNGF maturation of reducing mNGF degradation, might achieve a far more significant pro-cognitive effect. Even a symptomatic treatment extending good cognition for five years would translate to a 41% reduction in the global incidence of Alzheimer's dementia [78].

Emerging evidence suggests that cholinergic drugs might have some disease-modifying properties, albeit to a restricted extent, as we have discussed in a recent paper by the "Cholinergic System Working Group" [79]. To this effect, individuals with suspected prodromal Alzheimer's dementia taking donepezil show reduced rates of hippocampal, cortical, and BFCN atrophy [80, 81]. Conversely, aged individuals taking anti-cholinergic drugs have a higher incidence of Alzheimer's dementia and greater rates of hippocampal atrophy [82–85]. Furthermore, the degeneration of the BFCN precedes and predicts the degeneration of its target tissues in the entorhinal and cerebral cortices [86, 87]. It is possible that these effects are mediated by M1 AChR signalling in its capacity to reduce amyloidogenic processing of APP [88, 89]. Indeed, a combined M1 AChR/σ1 agonist has shown the ability to decrease amyloid pathology, attenuate CNS inflammation, and reverse cognitive deficits in a rat model of AD-like amyloid pathology [90]. As such, therapeutics capable of sustaining a fully functional cholinergic system further through the continuum of AD pathology and would therefore constitute a disease-modifying, although not curative, therapy. Such a therapy could perhaps target NGF dysmetabolism, as here demonstrated across the continuum of AD with relevance to cognitive outcomes.

Acknowledgements The authors express gratitude to the participants in the ROS study as well as to the team at the RUSH Medical Center for performing the cognitive testing and neuropathological analyses. The authors thank Dr. Daniel Lawrence from the Michigan Center for Integrative Research in Critical Care, USA for graciously providing the anti-neuroserpin antibody used in this study. The authors are also grateful for the revisions and suggestions provided by Drs Ezio Giacobini, Harald Hampel, and Giancarlo Pepeu on this manuscript. ACC acknowledges financial support from the Canadian Institutes of Health Research (CIHR) and the Alzheimer Society of Canada. He holds the McGill University Charles E. Frosst/Merck Chair in Pharmacology and is a member of the Canadian Consortium of Neurodegeneration in Aging. ACC wishes to thank Merck Canada for their unrestricted support. DAB was supported by grants P30AG10161 and R01AG15819 from the NIA. RP was the recipient of a Student Fellowship from the McGill Integrated Program in Neuroscience and CIHR Doctoral Award. MFI acknowledges support from a Bourse Postdoctorale from the Fonds de Recherche du Quebec Santé (FRQS). The funding bodies had no role in the design of the study or in the collection, analysis, and interpretation of data or in writing the manuscript.

Author contributions RP, MFI, and ACC conceived and designed the study. RP measured the proteins and transcripts in brain with guidance from MFI, AD, and ACC. DAB provided brain tissue as well as neuropathological and cognitive data. RP generated and analyzed the data. RP, MFI, and ACC wrote the manuscript. All authors have read and revised the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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