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Preservation of cortical sortilin protein levels in MCI and Alzheimer's disease

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Abstract

The nerve growth factor (NGF) precursor protein proNGF is the predominant NGF moiety found in the human neocortex and exhibits pro-apoptotic properties when bound to the p75^{NTR} neurotrophin receptor in the presence of sortilin, a Vps10p domain trafficking protein. Recently studies have shown that proNGF levels increase in the cortex of people who died with early stage Alzheimer's disease (AD) or with mild cognitive impairment (MCI), a putative prodromal AD stage. In contrast, cortical levels of the high-affinity, pro-survival NGF receptor TrkA are reduced in AD despite stable levels of p75^{NTR}. These data suggest a stoichiometric shift in proNGF and its receptors which favors proNGF binding of p75^{NTR}. Whether cortical levels of sortilin are altered during the progression of AD remains unknown. Therefore, we measured sortilin protein levels in postmortem superior frontal and superior temporal cortical tissue derived from Religious Orders Study subjects clinically diagnosed antemortem with no cognitive impairment (NCI), MCI or AD. No changes in frontal or temporal cortical sortilin protein levels occurred across the clinical groups. There was no association between sortilin levels and antemortem cognitive test scores. However, there was a positive association between temporal cortex sortilin levels and severity of neuropathology by Braak and NIA-Reagan diagnoses. The stability of cortical sortilin levels in the face of stable p75^{NTR}, increased proNGF, and reduced TrkA levels may favor pro-apoptotic proNGF:p75^{NTR}:sortilin trimeric interactions within the cortex during the earliest stages of AD. These findings are relevant to the development of NGF drug therapy for the treatment of dementia.

Keywords

Alzheimer's disease; mild cognitive impairment; proNGF; receptors; sortilin

INTRODUCTION

Although AD is characterized clinically by irreversible cognitive deterioration, evidence suggests that mild cognitive impairment (MCI) is one of the most common conditions affecting persons over the age of 65 and that the prevalence for MCI is more than double that of dementia

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[25]. Clinical pathobiologic studies suggest that MCI, in general, represents a prodromal or preclinical stage of AD [31]. Since it is believed that AD has an extensive preclinical phase, understanding the molecular pathogenesis underlying this disorder requires studying people during the early stages of the disease when brain pathology has been initiated prior to presentation of significant clinical symptoms [12]. Therefore, defining the cellular mechanisms underlying the selective vulnerability of neurons that degenerate during the progression of AD is important for the development of novel pharmacological therapies to arrest and/or slow the onset of this disease.

There is a widespread decline in several neurotransmitter containing cell bodies in AD and the most consistent loss is associated with long projection neurons, including cholinergic basal forebrain (CBF) neurons contained within the nucleus basalis. These neurons provide the major source of cholinergic innervation to the cerebral cortex and their degeneration plays a key role in memory and attention dysfunction in AD [2,23,26]. CBF neurons depend on the neurotrophic substance nerve growth factor (NGF) for survival and they express the high (TrkA) and low (p75^{NTR}) affinity NGF receptors required to bind this neurotrophin in cortical target fields. It has been hypothesized that dysregulation of NGF and its cognate receptors are key factors underlying CBF degeneration in AD [27].

NGF is synthesized as a precursor protein (proNGF) that is proteolytically cleaved to the mature biologically active neurotrophin peptide [13]. Mature NGF binds to the TrkA receptor, which activates signal transduction cascades mediating cell survival, [18] and to p75^{NTR}, which positively modulates NGF/TrkA binding [18]. However, proNGF is the predominant NGF moiety found in human brain [14], and several lines of evidence show that trimeric cell-surface interactions among proNGF, p75^{NTR} and the neurotensin receptor sortilin, a Vps10p domain trafficking protein, results in pro-apoptotic signaling [17,19,28,33,35].

In our ongoing investigations of the role that NGF/proNGF and its cognate receptors play in CBF neuron degeneration of during the progression of AD, we reported that in the cortex TrkA levels were reduced, p75^{NTR} levels remained stable, and proNGF levels were increased during disease progression [11]. Moreover, decreasing cortical TrkA levels and increasing proNGF levels were associated with poorer performance on cognitive tests such as the mini mental state exam (MMSE) [11,30]. Hence, we suggested that a reduction in cortical TrkA combined with increased proNGF levels result in enhanced binding between proNGF and p75^{NTR}, potentially shifting away from cell survival to apoptotic proNGF/p75^{NTR} signaling [9,25]. *In vitro* and *in vivo* studies suggest that the outcome of p75^{NTR} signaling in response to proNGF depends upon the presence of its binding partner, sortilin, which is required for apoptotic actions [16, 35]. However, to date there are no clinical pathological investigations of whether cortical sortilin levels are altered during the progression of AD or whether sortilin protein expression correlates with cognitive test scores and neuropathological criteria. Therefore, we examined cortical sortilin levels in tissue harvested from our on going clinical pathological study of participants in the Rush Religious Orders Study who died with an antemortem clinical diagnosis of no cognitive impairment (NCI), MCI or AD.

MATERIALS AND METHODS

Subjects

Brain tissues from 56 superior frontal (Brodmann area 10) and 29 superior temporal (Brodmann area 22) cortices were harvested; tissues were available from both regions in 27 of the cases examined. Subjects were participants in the Religious Orders Study, a longitudinal clinical-pathologic study of aging and AD in older Catholic nuns, priests and brothers [3,22]. Subjects were clinically categorized as NCI, MCI insufficient to meet criteria for dementia, or mild/moderate AD. The superior frontal MCI group contained 13 amnesic (aMCI) and 5 non-

amnesic (non-aMCI), whereas the superior temporal MCI group consisted of 9 aMCI and 2 non-aMCI cases. A summary of the study cohort (N=58) is shown in Table 1. The subset of 29 cases from which superior temporal tissues were collected showed demographic and clinical characteristics (data not shown) similar to those presented in Table 1 for the full cohort. Neuropathology among AD cases in this subset was less severe than that in the other AD cases (data not shown). The Human Investigations Committee of Rush University Medical Center approved the study.

Clinical Evaluation

Details of the clinical assessment of the Religious Orders Study cohort was published previously [3,22]. An annual clinical evaluation including an assessment for stroke and parkinsonian signs was performed. Neuropsychology technicians carried out a battery of cognitive tests, including the MMSE, Boston Naming, Word List Memory, Word List Recall and Word List Recognition and Logical Memory (Story A) [3,22]. Because neuropsychological tests do not measure cognition uniformly across different educational levels, cut-off points for rating impairment on each test were adjusted for education. A computer algorithm applied these cut-off points uniformly converting individual scores into impairment ratings in five cognitive domains (orientation, attention, memory, language and perception) [3,22]. An impaired score for each domain required deficits on multiple tests within that domain. A board-certified neuropsychologist summarized impairment data in each of the five cognitive domains as not present, possible, or probable. The neuropsychologist was blind to age, gender, race, clinical data other than education, occupation, sensory or motor deficits, and effort. A board-certified neurologist with expertise in geriatrics made a clinical diagnosis after review of clinical data from that year and examination of all subjects. The diagnosis of dementia and AD followed the recommendations of the joint working group of the National Institute of Neurological and Communicative Disorders and the Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) [20]. Although there are no clear consensus criteria for the clinical classification of MCI, the criteria used in this study were similar to, or compatible with, those used by others in the field to describe persons who were not cognitively normal, but did not meet accepted criteria for dementia [31]. Our MCI population is defined as those persons rated as impaired on neuropsychological testing by the neuropsychologist but who were not found to have dementia by the examining neurologist [3,22]. Postmortem interviews were carried out to determine medical conditions which occurred during the interval between the last clinical evaluation and death. A consensus conference of neurologists and neuropsychologists reviewed all available clinical data, and assigned a summary clinical diagnosis.

Pathological Evaluation

At autopsy, brains were processed as previously described [11,22]. Cases were excluded if they exhibited significant non-AD types of pathologic conditions (e.g., brain tumours, encephalitis, large strokes, multiple lacunar infarctions, Lewy body pathology, argyrophilic grains, tangle-only dementia). Brains were cut into 1-cm thick slabs using a Plexiglas jig. One hemisphere was immersion-fixed in 4% paraformaldehyde and the opposite hemisphere was snap-frozen in liquid nitrogen. Samples were stored at -80°C prior to biochemical analysis. From the immersion fixed brain slabs, brain regions were dissected, paraffin embedded, cut at $8\text{ }\mu\text{m}$, and stained with hematoxylin and eosin, Bielschowsky, thioflavin-S, and with an ubiquitin antibody. Neuropathologic designations of "normal" (with respect to AD or other dementing processes), "possible" or "probable AD", and "definite AD" were based on the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) a semi-quantitative estimation of neuritic plaque density, an age-adjusted plaque score, and presence or absence of dementia [21]. Each case also received Braak score based upon neurofibrillary tangle pathology [4] and a NIA-Reagan Diagnostic criteria [34].

Quantitative Immunoblotting

Brain samples were lysed on ice in lysis buffer (1.2% Triton X-100, 20 mM Tris-HCl, 140 mM NaCl, 10 mM EDTA pH 8.0) containing a protease inhibitor cocktail (Complete; Roche, Indianapolis, IN). The samples were centrifuged at $20,000 \times g$ and the protein concentration of the S1 supernatant subsequently determined using the Bicinchoninic Acid Kit (BCA-1, Sigma-Aldrich, St. Louis, MO). 75 μ g of brain extract was loaded for 4–16% SDS-PAGE, blotted to PVDF membranes and probed with a monoclonal anti-sortilin antibody (anti-neurotensin receptor 3, Cat. #612100, BD Biosciences, San Jose, CA). As a loading control the filters were subsequently re-probed with anti-synaptotagmin (BD Biosciences, Cat. #61034). Our previous quantitative immunoblotting study of synaptic protein expression during the progression of AD determined that this protein is unchanged by the disease process and can serve as an internal loading control [10]. Actin antiserum (Santa Cruz, CA) was also used in parallel experiments as an additional loading control to corroborate putative alterations in sortilin levels. Blots were developed and quantified using HRP-conjugated secondary antibodies (DakoCytomation; Glostrup, Denmark), ECL-plus (GE Healthcare; Hillerod, Denmark) and a Fujifilm LAS-1000P CCD imaging system (Vedbaek, Denmark). Sortilin immunoreactivity was normalized to synaptotagmin immunoreactivity on the same blots for quantitative analysis. In addition, three samples from healthy patients were present on all gels which served for normalization between blots. Samples were run in duplicate.

Statistical Analysis

A global cognitive score (GCS), which is a composite z-score of 19 measures of cognitive function [36], was computed for each case. For analysis we employed non-parametric methods, which are more robust to the influence of outliers. Demographic and neuropathological variables as well as sortilin levels were compared across clinical diagnostic groups by Kruskal-Wallis test or Fisher's exact test; pair-wise comparisons were performed with Bonferroni correction for multiple comparison. Associations between sortilin activity and demographic/neuropathological variables were assessed by Spearman rank correlation or Wilcoxon rank-sum test. The level of statistical significance was set at 0.05 (two-sided).

RESULTS

Case Demographics

The three clinical groups were comparable in age, sex, educational level, ApoE ϵ 4 allele prevalence, postmortem interval, and neuropathologic diagnosis (Table 1). For all subjects, the last clinical and cognitive evaluations were within the last 14 months of life. Subjects were clinically categorized as NCI ($n = 24$, mean $\pm$ SD for age = 82.8 ± 6.8 years, MMSE = 28.1 ± 1.5), MCI insufficient to meet criteria for dementia ($n = 27$, age = 83.6 ± 5.2 years, MMSE = 27.0 ± 2.4), or mild/moderate AD ($n = 33$, age = 86.3 ± 6.0 years, MMSE = 15.5 ± 7.7). MMSE and GCS scores were significantly lower in the AD group than in the MCI or NCI group; GCS scores were also significantly lower in MCI compared to NCI (pair-wise comparison, $p < 0.01$; Tables 1). Interestingly, ~50% of the cognitively intact NCI clinical diagnostic group displayed an intermediate to high likelihood of AD based on neuropathology alone (Table 1). Sortilin levels were not significantly different between the aMCI and non-aMCI in either the superior frontal or superior temporal groups.

Superior Frontal and Superior Temporal Cortex Sortilin Levels: Correlation with Neuropathologic and Cognitive Criteria

Sortilin protein levels did not differ across clinical groups in either of the cortical regions examined (Figure 1 and Table 2). Sortilin protein levels in superior frontal cortex were higher in the AD group (0.94 ± 0.30) compared with NCI and MCI respectively (0.83 ± 0.27 ; $0.83 \pm$

0.20). Levels in superior temporal cortex were lower in the NCI group (1.17 ± 0.56) compared with MCI and AD respectively (1.26 ± 0.47 ; 1.28 ± 0.48). These differences, however, did not reach statistical significance (Table 2). Although age and postmortem interval were not correlated among the cases examined, frontal cortex sortilin protein levels were found to be positively correlated with age (Spearman rank correlation, $r = 0.33$, $p = 0.014$) and negatively correlated with postmortem interval ($r = -0.38$, $p = 0.004$). There was no association between superior frontal sortilin protein levels and either cognitive scores or severity of neuropathology. On the other hand, superior temporal cortex sortilin protein levels were positively correlated with Braak scores ($r = 0.48$, $p = 0.0086$) and NIA-Reagan diagnosis ($r = 0.48$, $p = 0.0086$). No other clinical/neuropathologic variables showed an association with superior temporal cortical sortilin protein levels.

DISCUSSION

The present study found that cortical protein levels of the neurotensin receptor sortilin remained stable during the progression of AD. This finding supports a recent biochemical study showing that sortilin levels are unchanged in the hippocampus of control and AD patients [1]. Furthermore, sortilin co-expression with p75^{NTR} has been identified immunohistochemically in human CBF cortical projection neurons and no differences in sortilin levels were found between control and AD cases [8]. These findings are particularly important for understanding the complex nature of NGF signaling dysfunction and its relation to the highly vulnerable cholinergic cortical projection system in AD [29]. In this regard, recent findings indicate that the putative pro-apoptotic effect(s) of p75^{NTR}-mediated proNGF signaling is dependent on interactions between p75^{NTR} and sortilin [8,28], suggesting that sortilin acts as a proNGF co-receptor with p75^{NTR} [17]. For instance, *in vitro* studies show that sortilin expression is required for p75^{NTR}-mediated apoptosis following proNGF treatment [28], and blocking sortilin binding sites with neurotensin precludes the high-affinity binding of proNGF to p75^{NTR} and subsequent cell death [5,18,28,32]. Moreover, a recent report showed that the blockade of sortilin *in vivo* with neurotensin infusion abrogates proNGF-induced cell death in the aged rodent basal forebrain [1]. Thus, the impact of p75^{NTR} signaling in response to proneurotrophin binding may depend on the expression levels of its bound co-receptor sortilin, which acts as a cell-surface co-receptor with p75^{NTR} to mediate proNGF-induced pro-apoptotic signaling.

Pro-survival or pro-apoptotic signaling in CBF neurons during the onset of AD may depend upon fluctuations in the stoichiometry of TrkA, p75^{NTR}, proNGF and sortilin, and the physiological role of proNGF within these different milieus (e.g., decreased neurotrophism or increased apoptotic signaling) [9,24]. A shift in the ratio of any of these factors may alter the functional outcome that proNGF binding would impart upon CBF neurons during the development of AD. It is interesting that both cortical p75^{NTR} [11] and sortilin (present study) levels remain stable despite increased levels of proNGF [30] and decreased levels of TrkA [11] in CBF cortical projection sites during the progression of AD. Given the requirement of TrkA for NGF pro-survival signaling in CBF neurons, this shift in NGF receptor stoichiometry in the face of increased proNGF may favor the trimeric interactions of proNGF with p75^{NTR} and sortilin, thus activating pro-apoptotic pathways in cholinergic projection neurons during the early stages of AD. Whereas other studies indicate that proNGF can bind with less affinity to TrkA to induce a neurotrophic response [15,28], the lower levels of TrkA may not be sufficient to initiate proNGF induced cell survival signaling in AD. Furthermore, stable sortilin levels also occur despite increased levels of matrix metalloproteinase 9 (MMP-9), a protein involved in mature NGF degradation [6], during the progression of AD [7]. Together these data indicate the complex nature of the events underlying neurotrophin-mediated degeneration of the CBF projection system in AD.

Interestingly, we found no correlation between impairment on cognitive test scores and frontal and temporal cortical sortilin levels. This is markedly different from the association between increased proNGF [30] and MMP-9 levels and poorer cognitive test performance and the positive correlation between lower TrkA cortical levels and lower test scores [11] during the progression of AD. These findings suggest that sortilin expression is not a marker for the clinical progression of AD. However, superior temporal cortex sortilin levels were associated with Braak scores and NIA-Reagan diagnosis suggesting that the expression of this crucial cell survival protein is, in part, modulated by plaque and tangle pathology.

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Figure 1.

Cortical sortilin protein levels are stable during the progression of AD. A. Western blot shows strong sortilin immunoreactivity antibody in the cortex of a wild type (sortilin +/+) mouse, intermediate levels in a sortilin heterozygous mouse (+/-), and no reactivity in the cortex of a sortilin knockout mouse (-/-). B and C. Representative western blots and box plots showing sortilin and synaptotagmin immunoreactivity in the superior frontal (*left*) and superior temporal (*right*) cortex of subjects who died with a clinical diagnosis of no cognitive impairment (NCI), mild cognitive impairment (MCI), or Alzheimer's disease (AD). Synaptotagmin was used as the loading control for normalizing sortilin signals [10]. Box plot shows distribution of normalized mean values of cortical sortilin immunoreactivity among the NCI, MCI and AD subjects examined.

Table 1

Clinical, demographic, and neuropathological characteristics by diagnosis category

		Clinical Diagnosis			Total (N=58)	Comparison by diagnosis group
		NCI (N=17)	MCI (N=20)	AD (N=21)		
Age at death (years):	Mean ± SD (Range)	82.8 ± 6.8 (67–92)	83.6 ± 5.2 (72–92)	86.3 ± 6.0 (70–97)	84.3 ± 6.1 (67–97)	p = 0.2 ^a
Number (%) of males:		9 (53%)	11 (55%)	9 (43%)	29 (50%)	p = 0.8 ^b
Years of education:	Mean ± SD (Range)	18.4 ± 3.5 (12–25)	18.6 ± 3.6 (8–24)	17.5 ± 2.9 (8–24)	18.1 ± 3.3 (8–25)	p = 0.4 ^a
Number (%) with ApoE ε4 allele:		3 (18%)	4 (20%)	8 (38%)	15 (26%)	p = 0.4 ^b
MMSE:	Mean ± SD (Range)	28.1 ± 1.5 (25–30)	27.0 ± 2.4 (20–30)	15.5 ± 7.7 (0–25)	23.2 ± 7.6 (0–30)	p < 0.0001 ^{a*}
Global Cognitive Score (GCS) [§] :	Mean ± SD (Range)	0.56 ± 0.28 (–0.08, 1.15)	0.26 ± 0.32 (–0.25, 0.83)	–0.83 ± 0.55 (–2.22, –0.26)	–0.02 ± 0.72 (–2.22, 1.15)	p < 0.0001 ^{a**}
Post-mortem interval (hours):	Mean ± SD (Range)	5.0 ± 2.3 (2.3–11.0)	6.3 ± 3.7 (2.7–13.9)	6.5 ± 3.1 (2.7–12.4)	6.0 ± 3.1 (2.3–13.9)	p = 0.3 ^a
Distribution of Braak scores:	I–II III–IV V–VI No AD Low Intermediate High	4 12 1 0 7 9 1	3 15 2 0 8 11 1	1 8 12 0 4 8 9	8 35 15 0 19 28 11	p = 0.0008 ^{a*}
Distribution of NIA Reagan diagnosis (likelihood of AD):	No AD Low Intermediate High	0 7 9 2	0 8 9 7	0 4 8 2	0 19 28 13	p = 0.014 ^{a*}
Distribution of CERAD diagnosis (likelihood of AD):	No AD Low Intermediate High	4 2 9 2	7 1 6 6	2 1 8 10	4 23 18	p = 0.060 ^a

^a Kruskal-Wallis test.

^b Fisher's exact test.

* Pairwise comparisons with Bonferroni correction revealed that there was no significant difference between NCI and MCI, but both were significantly different from AD.

** Pairwise comparisons with Bonferroni correction showed significant difference between NCI, MCI, and AD.

[§] GCS was unavailable for 2 MCI and 3 AD cases.

Table 2
Summary of synaptotagmin-corrected sortilin levels in superior frontal (SF) and superior temporal (ST) cortex

		Clinical Diagnosis			Total	P-value ^a
		NCI	MCI	AD		
SF	Mean ± SD (Range) N	0.83 ± 0.27 (0.47–1.30) N=17	0.83 ± 0.20 (0.52–1.32) N=18	0.94 ± 0.30 (0.58–1.73) N=21	0.87 ± 0.26 (0.47–1.73) N=56	0.4
ST	Mean ± SD (Range) N	1.17 ± 0.56 (0.83–2.44) N=7	1.26 ± 0.47 (0.59–2.06) N=11	1.28 ± 0.48 (0.57–2.20) N=11	1.25 ± 0.48 (0.57–2.44) N=29	0.7

^aKruskal-Wallis test.