Declining levels of functionally specialized synaptic proteins in plasma neuronal exosomes with progression of Alzheimer’s disease

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ABSTRACT: Interactions of the presynaptic proteins, neuronal pentraxin 2 (NPTX2) and neuroligin 2α (NRXN2α), with their respective postsynaptic functional partners, GluA4-containing glutamate (AMPA4) receptor and neuroligin 1 (NLGN1), enhance excitatory synaptic activity in some areas of the hippocampus and cerebral cortex. As early damage of such excitatory circuits in the brain tissues of participants with Alzheimer’s disease (AD) correlates with cognitive losses, plasma neuron-derived exosome (NDE) levels of these 2 pairs of specialized synaptic proteins were quantified to assess their biomarker characteristics. The NDE contents of all 4 proteins were decreased significantly in AD dementia (n = 46), and diminished levels of AMPA4 and NLGN1 correlated with the extent of cognitive loss. In a preclinical period, 6–11 yr before the onset of dementia, the NDE levels of all but NPTX2 were significantly lower than those of matched controls, and levels of all proteins declined significantly with the development of dementia. Reductions in NDE levels of these specialized excitatory synaptic proteins may therefore be indicative of the extent of cognitive loss and may reflect progression of the severity of AD.—Goetzl, E. J., Abner, E. L., Jicha, G. A., Kapogiannis, D., Schwartz, J. B. Declining levels of functionally specialized synaptic proteins in plasma neuronal exosomes with progression of Alzheimer’s disease. FASEB J. 32, 000–000 (2018). www.fasebj.org

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Diminished synaptic function and loss of synapses are characteristic early elements of the neuropathology of Alzheimer’s disease (AD), usually attributed to the neuronal deposition of neurotoxic amyloid-β (Aβ) peptide oligomers (1, 2). The distribution and extent of brain synaptic pathology in postmortem brain tissues of participants with AD correlate generally with the severity of pre-mortem cognitive loss (3, 4). Our initial analyses of plasma neuron-derived exosome (NDE) content of several synaptic proteins in AD demonstrated lower levels—similar to decreases in postmortem AD brain tissues—compared with those of matched controls. In cross-sectional studies, the plasma NDE levels of the presynaptic proteins, synaptotagmin and synaptophysin, and of the postsynaptic proteins, synaptopodin and neurogranin, in patients with AD were significantly lower than those for controls, whereas plasma NDE levels of the synaptic membrane protein, GAP43, were only marginally lower in patients with AD than in controls (5). The same synaptic proteins in NDEs from the plasma of participants who were initially cognitively normal but who subsequently developed definite AD dementia were at significantly lower preclinical levels than in plasma NDEs of matched controls (5). There was also a progressive decline of plasma NDE levels of synaptotagmin, synaptopodin, and GAP43, but not of synaptophysin or neurogranin, in these participants 2–10 yr later at the time of diagnosis of AD dementia.

Synaptic proteins that were investigated in our first study are widely distributed in CNS synapses and share functional properties of associating with some other synaptic proteins to form complexes that are capable of binding calcium, regulating synaptic calcium concentration, and controlling vesicle fusion, recycling, and readily releasable pool size (5). Two classes of proteins that have essential synaptic maintenance functions largely localized in excitatory circuits, rather than those of the widely distributed cluster studied by us originally, are also observed at lower levels in the postmortem brain tissues of patients with AD than in controls, and their losses appear to contribute directly to the pathogenesis of AD (6–12).
Neuronal pentraxin 2 (NPTX2) complexes that include NPTX1 and NPTX receptors are expressed presynaptically and secreted by the excitatory synapses of pyramidal neurons of the hippocampus and cerebral cortex, where they bind specifically with the GluA4-containing glutamate (AMPA4) receptors on fast-spiking parvalbumin interneurons and thereby strengthen these excitatory synapses (9, 13). Decreased levels of NPTX2 and correspondingly diminished levels of AMPA4 in the brain tissues of patients with AD and mice with models of AD are associated with altered pyramidal neuron excitability (14). Presynaptic neurexin2α (NRXN2α) and the postsynaptic adhesion protein, neuroligin1 (NLGN1), interact trans-synaptically to ensure structural stability and functions of excitatory synapses in the hippocampus and cortex (6, 7). NLGN1 and NRXN2α both bind synaptotoxic Aβ peptide oligomers to increase their synaptic concentrations, thereby enhancing oxidative stress and promoting synaptic damage in AD (15–17). Cognitive loss induced by the administration of Aβ peptide oligomers to mice is lessened by concurrent doses of Abs to NLGN1 and NRXN2α that diminish Aβ peptide oligomer binding by NLGN1 and NRXN2α (17).

Here, we report significantly lower levels of NPTX2, AMPA4, NLGN1, and NRXN2α in the plasma NDEs of patients with AD compared with those of matched controls as well as the striking progression of such diminished levels from those at a time of normal cognition to preclinical AD to those at the time of the development of AD dementia.

MATERIALS AND METHODS

Experimental design and patient evaluation

For cross-sectional studies, we retrospectively identified 28 patients with early AD—mild cognitive impairment or mild dementia—who had been evaluated extensively in the Clinical Research Unit of the National Institute on Aging (NIA; Baltimore, MD, USA) and 28 age- and gender-matched controls who had donated blood at the Jewish Home of San Francisco (JHSF) during the same period of time as the patients (Table 1). For longitudinal studies, we identified 3 patients from the University of Kentucky Sanders-Brown Center on Aging, and 15 patients from JHSF with moderate AD who had provided blood at 2 times: first, when cognitively intact (AD1; Table 1), and again 6–11 yr later, after diagnosis of dementia (AD2; Table 1). Eighteen cognitively normal controls who were age and gender matched with the AD1 group were found at JHSF, and their plasmas were obtained in the same time period. One investigator (E.J.G.) supervised the identification and storage of all plasmas by the same procedures. Plasmas from patients in the longitudinal studies were analyzed without knowledge of the clinical diagnosis.

Patients with AD had mental status testing at the time of each blood sampling, Mini-Mental State Examination and the AD Assessment Scale-cognitive subscale were conducted as described previously (18). Cross-sectional patients from the NIA had amnestic mild cognitive impairment or mild dementia with a high probability of AD and a Clinical Dementia Rating global score of 0.5 or 1.0 according to NIA–Alzheimer’s Association and Informal Working Group (IWG)-2 criteria (19, 20). Each patient in the cross-sectional study had a CSF level of Aβ1–42 of <192 pg/ml and an elevated CSF level of P-T181-tau, which supported their diagnosis of AD (21). AD1/AD2 patients from JHSF and the University of Kentucky had probable AD and mild-to-moderate dementia at the AD2 stage by National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS/ADRDA) criteria and had a Clinical Dementia Rating global score of 1.0 at the time of the second blood collection (22).

The performance and procedures of all studies were approved by the respective institutional review committees of participating medical centers.

| TABLE 1. Characteristics of patients with AD and control participants |
|---------------------------|-----------|----------------|----------------|----------------|
|                          | Total     | Age            | MMSE           | ADAS-cog       |
|                          | n         | Male/female    |                |                |
| Cross-sectional sets     |           |                |                |                |
| C                        | 28        | 12/16          | 73.2 ± 1.47    | 29.7 ± 0.13    | 3.32 ± 0.31    |
| AD                       | 28        | 12/16          | 73.1 ± 1.44    | 25.6 ± 0.83*   | 13.7 ± 1.31*   |
| Longitudinal sets        |           |                |                |                |
| C                        | 18        | 10/8           | 70.1 ± 1.66    | 28.3 ± 0.96    | 3.68 ± 0.45    |
| AD1                      | 18        | 10/8           | 69.4 ± 1.71    | 28.7 ± 0.47    | 4.19 ± 0.57    |
| AD2                      | 18        | 10/8           | 78.2 ± 1.75    | 20.2 ± 1.50*   | 17.6 ± 1.64*   |

AD and C are the patients and controls in the cross-sectional study of AD. AD1 and AD2 are the groups of patients with AD who were evaluated at 2 times in the longitudinal study, at a preclinical phase and after conversion to moderate dementia, respectively, and C is the controls matched to AD1 patients. ADAS-cog, AD Assessment Scale-cognitive subscale; MMSE, Mini-Mental State Examination. The significance of differences between cognitive state (MMSE and ADAS-cog) values of the groups were calculated by an unpaired Student’s t test for C vs. AD (cross-sectional sets) and for C vs. AD1 and by a paired Student’s t test for AD1 vs. AD2 (longitudinal sets). Data represent the means ± SEM. *P < 0.001.
Enrichment of plasma NDEs for extraction and ELISA quantification of proteins

Aliquots (0.25 ml) of plasma were incubated with 0.1 ml thrombolastin D (Thermo Fisher Scientific, Waltham, MA, USA), followed by the addition of calcium- and magnesium-free Dulbecco's balanced salt solution with protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail (Thermo Fisher Scientific) (5). After centrifugation at 3000 g for 30 min at 4°C, NDEs were harvested from the resultant supernatants by sequential ExoQuick (System Biosciences, Mountain View, CA, USA) precipitation and immunochemical enrichment with mouse anti-human CD171 (LICAM neural adhesion protein) biotinylated Ab (clone 5G3; ebiosciences, San Diego, CA, USA) as previously described (5, 23, 24). M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Life Sciences) lysates of NDEs that contained protease and phosphatase inhibitors were stored at −80°C. Astrocyte-derived exosomes (ADEs) were isolated as described previously (25) from plasmas of the same participants who provided the NDEs.

NDE and ADE proteins were quantified by ELISA kits for human tetraspanning exosome marker CD81 (American Research Products–Cusabio, Waltham, MA, USA); NPTX2 and AMPA4 (American Research Products–Cloud-Clone Corp.); and NRXN2a and NLGN1 (American Research Products—Qayee-Bio).

Mean values for all determinations of CD81 in each assay group was set at 1.00 and relative values of CD81 for each sample were used to normalize their recovery. The selective representation of the present set of synaptic proteins in NDEs, in contrast to ADEs, was demonstrated by concurrent analyses of NLGN1 and NRXN2α in both types of exosomes from plasmas of a subset of 20 control participants for the cross-sectional study. The respective levels of NLGN1 and NRXN2α were (means ± SEM) 7548 ± 923 and 3921 ± 349 pg/ml for ADEs, and 174,226 ± 18,073 and 78,519 ± 7736 pg/ml for NDEs.

Statistical analyses

The Shapiro-Wilks test demonstrated that data in all sets were distributed normally. The statistical significance of differences between means for longitudinal groups AD1 and AD2 were determined by using paired Student’s t test (Prism 6; GraphPad Software, La Jolla, CA, USA). Relationships between NDE content of a cargo synaptic protein and the corresponding cognitive score of a patient with AD were evaluated by Pearson correlation coefficients.

RESULTS

Patients with AD in the cross-sectional study had cognitive scores consistent with mild cognitive impairment or mild dementia that were significantly different from the normal range of scores for controls (Table 1). The longitudinal study participants who were evaluated initially at their AD1 pre-clinical phase had normal cognitive scores that were not different than those of their controls (Table 1). At the time of donation of the second blood sample, the longitudinal group was termed AD2 and had mild-to-moderate dementia and significantly worse cognitive scores than at the AD1 phase.

NDE levels of both synaptic proteins of the 2 sets were significantly lower than those of matched controls (Figs. 1 and 2). Values for the NLGN1–NRXN2a pair were higher than those of the AMPA4–NPTX2 pair and displayed much less overlap with control values. For NLGN1 and NRXN2a, only 4 and 5 control participant values, respectively, were in the range of those for patients with AD (Fig. 2). There were significant inverse correlations between elevated AD Assessment Scale-cognitive subscale scores and decreased NDE levels of AMPA4 and NLGN1, but not of NPTX2 or NRXN2a (Fig. 3). Similarly, there were significant positive correlations between depressed Mini-Mental State Examination scores and decreased NDE levels of AMPA4 (r = 0.621; P = 0.0094) and NLGN1 (r = 0.525; P = 0.0053), but not of NPTX2 or NRXN2a. No correlations were observed between reduced levels of CSF Aβ1-42 and decreased levels of any of the synaptic proteins. One half of patients with AD in the cross-sectional study were being treated with 5 or 10 mg/d donepezil, but there were no significant differences between levels of NDE synaptic proteins for treated and untreated subsets.

Figure 1. NDE levels of AMPA4 and NPTX2 in cross-sectional control and AD groups. Each point represents the value for a control participant or patient with AD, and the horizontal line in point clusters is the mean level for that group. Control and AD patient values are 2276 ± 180 and 766 ± 68.0 pg/ml (means ± SEM), respectively, for AMPA4 and 2656 ± 343 and 1250 ± 123 pg/ml, respectively, for NPTX2. The significance of differences between values for controls and patients with AD was calculated by using an unpaired Student’s t test. *P < 0.01, **P < 0.0001.
For the longitudinal series of patients with AD, NDE levels of AMPA4, NLGN1, and NRXN2α, but not NPTX2, were significantly lower than those of matched controls in the AD1 preclinical phase (Fig. 4). At the AD2 stage of mild-to-moderate dementia 6–11 yr later, NDE levels of all 4 synaptic proteins had decreased significantly for the group and in every patient compared with their levels at the AD1 phase.

**DISCUSSION**

The methods described here permit the quantification of meaningful levels of both members of the 2 sets of excitatory synaptic proteins as well as the demonstration of significant differences between levels in patients with AD and controls and between preclinical and clinically apparent stages of AD (Figs. 1, 2, and 4). There are 4 major differences between the present results and the findings for the group of broadly distributed synaptic proteins previously reported (5).

The first is distinctive functions in specific excitatory synapses of the hippocampus and areas of the cerebral cortex. Presynaptic complexes that include NPTX2 are secreted into the excitatory synapses of pyramidal neurons of the hippocampus and cerebral cortex, bind specifically with AMPA4, and thereby mediate enhanced synaptic transmission in these circuits. Presynaptic NRXN2α and the postsynaptic adhesion protein, NLGN1, interact transsynaptically in these excitatory synapses of the hippocampus and cortex to also ensure structural stability and enhanced synaptic function. The second distinguishing feature of these 2 protein pairs is their localization in areas that are affected very early in AD, where the NRXN2α–NLGN1 pair also may directly contribute to pathogenesis via binding and the selective concentration of neurotoxic oligomers of Aβ peptides, such as Aβ1–42 (17).

The third difference between 2 of these functionally specialized synaptic proteins and numerous other NDE cargo proteins that have been implicated in AD is the correlation between cognitive scores and the levels of AMPA4 and NLGN1 (Fig. 3). This type of correlation, that suggests value for NDE levels of these proteins as indicators of AD clinical severity, is shared only by the more broadly distributed synaptic proteins synaptopodin, synaptotagmin, and synaptophysin, but not by a wide range of other NDE cargo proteins (5). Finally, the fourth distinguishing feature of
these 2 synaptic protein pairs is a striking progressive decrease in all NDE levels as patient clinical status declines from normal cognition in the preclinical stage to dementia with overt AD (Fig. 4). This progressive reduction in NDE level with declining clinical status was observed only for a synaptotagmin, GAP43, and to a much lesser extent for synaptopodin, but not for synaptophysin or neurogranin, of the more broadly distributed set of synaptic proteins (5).

In our prior study of NDE levels of a more broadly distributed set of synaptic proteins, decreases for 16 patients with frontotemporal dementia (FTD) compared with those of matched controls approached the magnitude of decreases that were observed for patients with AD only for one synaptotagmin, GAP43, and to a much lesser extent for synaptopodin, but not for synaptophysin or neurogranin, of the more broadly distributed set of synaptic proteins (5).

Several variables may affect the laboratory results reported here. Diminished NDE levels of cargo proteins reflect lower neuronal concentrations of these proteins, but may also be influenced by less efficient loading of some proteins into NDEs as the disease progresses. There also is the potential involvement of altered postloading proteolysis. Interpretation of the results is also subject to several limitations. One such issue is sample size, such that findings may not be generalizable to larger and different populations. Nonetheless, the findings reported here advance our understanding of which synaptic proteins may be affected in early AD. The possibility of establishing this new set of specialized excitatory synaptic proteins as useful biomarkers of the stage and severity of AD will
depend on additional analyses of NDE trafficking and protein handling, as well as clinical investigations of larger groups of patients over longer periods of time.

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AUTHOR CONTRIBUTIONS

E. J. Goetzl developed the initial concept and approach; E. J. Goetzl and J. B. Schwartz designed the study; E. J. Goetzl performed the exosome isolations and ELISAs; E. J. Goetzl and J. B. Schwartz selected and evaluated the patients and control participants; and E. J. Goetzl, E. L. Abner, D. Kapogiannis, and J. B. Schwartz prepared and edited the manuscript.

REFERENCES


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