1   **Associations between fluid biomarkers and PET imaging ([11C]UCB-J) of synaptic pathology in**

2   **Alzheimer's disease**

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29

30 **KEYWORDS**

31 Alzheimer's disease, synaptic pathology, mass spectrometry, biomarkers, SV2A

32

33 **ABBREVIATIONS**

34 AP2B1, adaptor related protein complex 2 subunit beta 1; AD, Alzheimer's disease; Aβ, amyloid-β;

35 $A\beta_{42/40}$, Amyloid-β 42/40; CDR, Clinical Dementia Rating; CSF, cerebrospinal fluid; CV, coefficient

36 of variation; MMSE, Mini-Mental State Examination; NPTX, neuronal pentraxin; PEBP-1,

37 phosphatidylethanolamine-binding protein 1; PET, positron emission tomography; P-tau$_{181}$, tau

38 phosphorylated at Thr181; GDI-1, rab GDP dissociation inhibitor alpha; ROI, region of interest; PVC,

39 partial volume correction; r, Pearson's rank correlation coefficient; SV2A, synaptic vesicle

40 glycoprotein 2A; T-tau, total tau.

41 **ABSTRACT**

42 **INTRODUCTION:** Positron Emission Tomography (PET) imaging with ligands for synaptic vesicle

43 glycoprotein 2A (SV2A) has emerged as a promising methodology for measuring synaptic density in

44 Alzheimer's disease (AD). We investigate the relationship between SV2A PET and CSF synaptic

45 protein changes of AD patients.

46 **METHOD:** Twenty-one participants with early AD and 7 cognitively normal (CN) individuals

47 underwent [^{11}C]UCB-J PET. We used mass spectrometry to measure a panel of synaptic proteins in

48 CSF.

49 **RESULTS:** In the AD group, higher levels of syntaxin-7 and PEBP-1 were associated with lower

50 global synaptic density. In the total sample, lower global synaptic density was associated with higher

51 levels of AP2B1, neurogranin, γ-synuclein, GDI-1, PEBP-1, syntaxin-1B, and syntaxin-7 but not with

52 the levels of the neuronal pentraxins or 14-3-3 zeta/delta.

53 **CONCLUSION:** Reductions of synaptic density found in AD compared to CN participants using

54 [^{11}C]UCB-J PET were observed to be associated with CSF biomarker levels of synaptic proteins.

55

56

BACKGROUND

Synaptic dysfunction and degeneration are an early and significant part of the pathology occurring in Alzheimer's disease (AD) as well as in other neurodegenerative diseases [1]. Postmortem brain studies have described a widespread reduction in synapse numbers in AD in comparison to healthy individuals and this synaptic loss is the major structural correlate of cognitive decline, even more so than amyloid-beta (Aβ) plaque pathology [2]. These findings have contributed to an increasing interest in the development and implementation of synaptic biomarkers in diagnostics as well as mechanistic insight into synaptic pathology.

Already 30 years ago, the first studies emerged verifying the presence of synaptic proteins in the cerebrospinal fluid (CSF). Since then, several methods for the quantification of a wide range of synaptic proteins in living patients have been developed as synaptic biomarkers [3]. One of the most established synaptic biomarkers is neurogranin, a postsynaptic protein that is specifically increased in AD with changes at early stages of cognitive impairment [4] and even before symptom onset [5]. Other synaptic proteins including SNAP-25, synaptotagmin-1, and GAP43 increase along with biomarkers of cerebral amyloid pathology in symptomatic and asymptomatic disease, and are considered AD specific (*e.g.,*) [6-8]. Interestingly, some synaptic proteins are lower in CSF samples of participants with neurodegenerative disease. For example, neuronal pentraxins have decreased levels across symptomatic neurodegenerative diseases, including AD, compared to healthy individuals and the levels of pentraxins strongly correlate with cognitive decline [9-11]. Emerging evidence thus points to the fact that synaptic pathology mechanisms are more complex than earlier believed and that all synaptic proteins may not represent the same pathological pathways.

Recent progress in synaptic positron emission tomography (PET) imaging has also allowed for the evaluation of synaptic alterations *in vivo* [1, 12]. Utilizing [^{11}C]UCB-J, a PET tracer that binds the synaptic vesicle glycoprotein 2A (SV2A) which is expressed in nearly all synapses, the synaptic density of patients with AD was found to be reduced in the medial temporal and neocortical brain regions compared with healthy controls [12, 13]. SV2A PET has been used to characterize the patterns of synaptic loss due to neurodegenerative disease and early studies have described complex

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84   relationships with core AD biomarkers such as greater synaptic los in areas of tau deposision [14],

85   significant correlations between synaptic density and metabolism measured with [^{18}F]FDG [15], and

86   stage dependent correlations between hippocampal synaptic density and global amyloid measured with

87   [^{11}C]PiB [16]. In addition, SV2A PET has been used to demonstrated significant synaptic loss in

88   dementia with Lewy bodies [17], frontotemporal dementia [18], Parkinson's disease [19], and

89   Huntington's disease [20]. Thus, both imaging and CSF protein biomarkers are altered in AD and

90   other neurodegenerative disorders and these changes are likely reflect synaptic pathology. However,

91   the meaning of synaptic protein concentration changes measured in vivo with PET or CSF assays

92   remains unclear. These changes may reflect altered clearance or altered protein production and

93   secretion into the CSF due to synapse degeneration or changes in synaptic activity [21].

94   In the present study, we evaluate the relationship between SV2A PET and CSF synaptic protein

95   changes of AD patients to gain insight into AD synaptic pathology and the meaning of synaptic

96   protein biomarkers. To do so, we have measured a panel of synaptic proteins in a small cohort of AD

97   patients and control participants who also have undergone SV2A PET.

98   **METHOD**

99   *Study design and population*

100   The requirement of participation included fulfilling the diagnostic criteria for amnestic mild cognitive

101   impairment (MCI, n=5) [22] or probable dementia due to AD (n=16) [23]. AD and MCI participants

102   were additionally required to have a positive PET scan with [^{11}C]Pittsburgh Compound B ([^{11}C]PiB), a

103   Mini-Mental State Examination (MMSE) score <26 and 24-30, respectively, and a Clinical Dementia

104   Rating (CDR) score of 0.5-1.0 and 0.5, respectively. Cognitively normal participants (CN, n=7) were

105   required to have a negative [^{11}C]PiB PET scan, MMSE score <26, and a CDR score of 0. All

106   participants gave written informed consent approved by the Yale University Human Investigation

107   Committee.

108   *Brain imaging*

6

109 To define regions of interest (ROIs) and perform partial volume correction (PVC) using the iterative

110 Yang approach [24], T1-weighted magnetic resonance imaging (MRI) was performed. FreeSurfer

111 [version 6.0] was used for volumetric segmentation and cortical reconstruction. A high-resolution

112 research tomograph (207 slices, resolution <3 mm, full width at half maximum (FWHM)) was used

113 for the PET scans with event-by-event motion correction. Dynamic [11C]PiB and [11C]UCB-J scans

114 60 minutes, following administration of a bolus of up to 555 and 740 MBq of tracer, respectively. As

115 previously described, SRTM2 (cerebellum reference region) was used with dynamic scan data from 0

116 to 60 min to generate parametric images of [11C]PiB BP_{ND} and [11C]UCB☐J DVR [24].

117 *LC-MS analysis*

118 CSF samples were collected in polypropylene tubes by lumbar puncture, centrifugated (2200 x g for

119 10 min, 20°C), and stored at -80°C. Eleven synaptic proteins were analyzed in the synaptic panel

120 analysis; neurogranin, γ-synuclein, the activating protein 2 subunit complex beta (AP2B1), rab GDP

121 dissociation inhibitor alpha (GDI-1), phosphatidylethanolamine-binding protein 1 (PEBP-1), 14-3-3

122 ζ/δ, syntaxin-1B, syntaxin-7, and the neuronal pentraxins (-1 (NPTX1), -2 (NPTX2), and the receptor

123 (NPTXR)). The sample preparation of 100 µL of CSF samples entailed the addition of internal

124 standard (mix of stable-isotope-labeled peptides, 25 µL, 0.032 pmol/µL), reduction, alkylation, tryptic

125 digestion, and solid-phase extraction, (for thorough protocol, refer to [25]). Quantification of the

126 synaptic proteins was performed on a micro-high-performance liquid chromatography-mass-

127 spectrometry system (6495 Triple Quadrupole LC/MS system, Agilent Technologies) equipped with a

128 Hypersil Gold reversed-phase C18 column (dim.=100x2.1 mm, particle size=1.9 µm, Thermo Fisher

129 Scientific), for detailed settings see Suppl. Table 1. Assay performance was evaluated during the run

130 by injections at regular intervals of a quality control sample.

131 *Data processing and statistical analysis*

132 Skyline 20.1 (MacCoss Lab Software) was utilized for peak inspection and adjustment of the

133 chromatographic spectra and R software was used for the statistical analysis. For the proteins for

134 which more than one peptide was analyzed, the peptide with the best repeatability (lowest CV), was

135 chosen for the statistical analysis (Suppl. Table 2). The group comparisons of the demographic

136 characteristics and biomarkers were evaluated by analysis of variance (ANOVA) and chi-square

137 goodness of fit test for continuous and categorical variables, respectively. Associations between

138 synaptic density and CSF biomarker levels were explored with Pearson's rank correlation analysis and

139 brain maps for visualization were created by setting each brain region's voxels uniformly to the

140 calculated effect size (Pearson's *r*).

141 **RESULTS**

142 *Participant characteristics and biomarker levels*

143 The study sample was well-balanced regarding age and sex and the AD participants had both typical

144 clinical characteristics (MMSE=23.0±2.9) and core CSF biomarker levels of total tau (t-tau), tau

145 phosphorylated at Thr181 (p-tau$_{181}$), amyloid-β 42/40 (Aβ$_{42/40}$) (Table). Out of the 11 synaptic proteins

146 quantified 6 proteins (neurogranin, γ-synuclein, GDI-1, PEBP-1, 14-3-3 ζ/δ, and syntaxin-1B) showed

147 significantly higher protein levels (p<0.05) in the AD group compared to CN.

148 *Association between synaptic density and synaptic proteins*

149 The primary analysis investigated the association between global synaptic density (*DVR*) in a

150 composite of AD-affected regions [24] and synaptic protein levels in the AD group. We also examined

151 the same associations in the the CN group and the total sample (Suppl. Table 3). In the AD group,

152 significantly higher levels of syntaxin-7 (*r*=-0.49, p-value=0.024) and PEBP-1 (*r*=-0.47, p-

153 value=0.033) were associated with lower global synaptic density. In the total sample, lower global

154 synaptic density was significantly associated with higher levels of AP2B1, neurogranin, γ-synuclein,

155 GDI-1, PEBP-1, syntaxin-1B, and syntaxin-7 (*r*=-0.40 to -0.54, p-value<0.05) but not with the levels

156 of the neuronal pentraxins or 14-3-3 zeta/delta. In the CN group, there were no significant associations

157 between any proteins and global synaptic density. When PVC was performed on PET data, in the AD

158 group, only significantly higher levels of syntaxin-7 (*r*=-0.51, p-value<0.05) were associated with

159 lower PVC global synaptic density. In the total sample, lower global synaptic density was significantly

160 associated with higher levels of AP2B1, GDI-1, and PEBP-1 (*r*=-0.40 to -0.44, p-value<0.05). In the

161 CN group, there were no significant associations between any proteins and PVC global synaptic

8

162   density. When additional analyses assessed the association between CSF protein biomarkers and

163   synaptic density in all brain regions, negative associations were found across many parietal, temporal,

164   prefrontal, and occipital cortical regions (data not shown). The associations varied between biomarkers

165   as seen by syntaxin-7, which showed the strongest associations with synaptic density in different brain

166   regions, and neuronal pentraxin-2, which showed no associations with synaptic density as measured

167   (Figure). Notably, in the context of AD where the hippocampus is known to be an area of early

168   synaptic loss, there were no significant associations between any synaptic proteins and hippocampal

169   synaptic density in either the separate groups or total sample (Figure and Suppl. Table 4).

170

171   **DISCUSSION**

172   In this study, we examined the relationship between potential biomarkers of synaptic pathology and

173   synaptic density using a mass spectrometric synaptic protein panel and [^{11}C]UCB-J PET. In the AD

174   group, the most robust global associations were seen for syntaxin-7 and PEBP-1. While in the total

175   sample, associations were found for several of the synaptic proteins, including the established synaptic

176   biomarker neurogranin. Interestingly, no associations were found for the neuronal pentraxins, synaptic

177   proteins that are emerging as interesting biomarkers in neurodegenerative diseases.

178   Syntaxin-7 is involved in vesicle endocytosis at the synapse, as a SNARE protein, and thus mediation

179   of endocytic trafficking [26]. Endocytic impairment has been implicated to be a feature of many

180   neurodegenerative diseases, not the least in AD [27]. Studies of syntaxin-7 as a potential biomarker

181   have however shown no changes in the CSF of AD patients or other diseases compared to controls [9,

182   25]. We also observed a non-significant difference between AD patients when compared to controls in

183   the current study. However, syntaxin-7 was shown to have the strongest association with synaptic

184   density as quantified by [^{11}C]UCB-J PET within AD patients. Since SV2A, the target for [^{11}C]UCB-J,

185   and syntaxin-7 are both synaptic vesicle proteins the stronger associations between synaptic density

186   and syntaxin-7 compared to the other synaptic proteins might be attributed to the proximity in function

187   between the two. PEBP-1 was also associated with [^{11}C]UCB-J PET in AD patients, but displayed

188   higher levels in the AD compared to the CN group. PEBP-1 is a regulatory protein with modulatory

189   roles in several protein kinase signaling cascades as well as the precursor to the hippocampal

190   cholinergic neurostimulating peptide (HCNP) implicated in the induction of acetylcholine synthesis

191   and enhancement of glutamatergic activity [28, 29].

192   The neuronal pentraxins are three synaptic proteins, two secreted glycoproteins (NPTX1 and NPTX2),

193   and their plasma membrane-anchored receptor (NPTXR), which have important roles in synaptic

194   function and plasticity [30]. By associating and forming heteromultimers the neuronal pentraxins are

195   involved in the recruitment and localization of neurotransmitter receptors to the postsynaptic

196   membrane during receptor exocytosis. As previously mentioned, pentraxins have been recognized as

197   potential synaptic biomarkers with decreased levels across neurodegenerative diseases compared to

198   healthy individuals and whose levels strongly correlate with cognitive decline [9, 10, 31-36].

199   However, in the current study, neuronal pentraxins levels in the CSF did not differ between AD and

200   CN groups, and did not correlate with reduced synaptic density in AD. The cause of this inconsistency

201   is not clear, but differences in AD pathologic stage or other participant characteristics between sample

202   studies could be contributing. In combination with the observation that the levels of the neuronal

203   pentraxins in the CSF of AD patients differ in the directionality of change (decreased instead of

204   increased) compared with most other synaptic proteins might further implicate that the neuronal

205   pentraxins levels in CSF are indicative of a unique synaptic pathologic processes occurring in AD.

206   Further studies are needed to confirm these findings and to investigate what possible mechanisms

207   might underlie the difference between synaptic protein alterations in AD.

208   The main limitation of this study is the modest sample size leading to limited power of the study to

209   detect associations. This might have especially affected the control group where no associations were

210   seen, nevertheless, all scatter plots (data not shown) were visually inspected and no convincing

211   associations were observed. However, we are encouraged that several proteins including syntaxin-7

212   and PEBP-1 demonstrated associations with [^{11}C]UCB-J PET in AD. Future studies with larger

213   sample sizes and a broader range of synaptic proteins may permit a better understanding of the

214   relationship between altered synaptic density fluid synaptic biomarkers in AD.

215   **CONCLUSIONS**

216   We observed that the reductions of synaptic density found in AD compared to CN participants using

217   [^{11}C]UCB-J PET are associated with biomarker levels of synaptic proteins.

218   **Ethics approval and consent to participate**

219   **Consent for publication**

220   Not applicable

221   **Availability of data and materials**

222   Derived data supporting the findings of this study are available from the corresponding author on

223   request, providing data transfer is in agreement with the participating centre national legislation and

224   institutional review centre.

225   **Competing interests**

226   HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Annexon,

227   AZTherapies, CogRx, Denali, Eisai, Nervgen, Pinteon Therapeutics, Red Abbey Labs, Roche,

228   Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia

229   sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker

230   Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB has

231   served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon,

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Authors' contributions

262 JN, NJA, and APM designed the study. JN performed the experiments. JN, NJA, ES, RSO and APM

263 analysed the data and wrote the manuscript. AB, HZ, KB were responsible for supervision,

264 conceptualization, and verification of the underlying data. APM, RSO, REC and CHvD provided the

265 CSF samples of the clinical cohort. APM, RSO and CHvD participated in diagnosis of the patients and

266   CSF samples collection. AB, ES, REC, CHvD, HZ, and KB contributed to the interpretation of the

267   results and provided critical feedback of the manuscript. All authors have reviewed the manuscript.

268

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365

366 **FIGURE LEGENDS:**

367 **Figure.** Regional associations (Pearson's *r*) between cerebrospinal fluid syntaxin-7 or neuronal

368 pentraxin-2 (NPTX2) and synaptic density (*DVR*) determined by [^{11}C]UCB-J PET in Alzheimer's

369 disease (AD) and cognitively normal (CN) participant groups. Analysis was performed with and

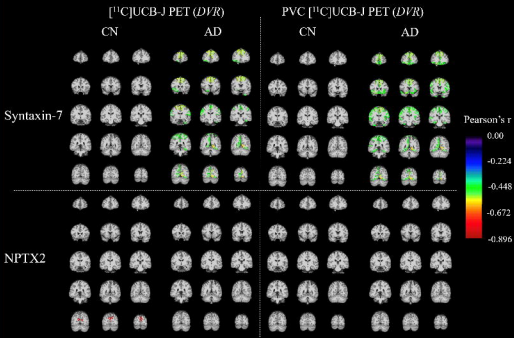370 without partial volume correction (PVC) of PET data.

Syntaxin-7

NPTX2

[¹¹C]UCB-J PET (*DVR*)

PVC [¹¹C]UCB-J PET (*DVR*)

CN   AD

CN   AD

Pearson's r

0.00
-0.224
-0.448
-0.672
-0.896

Table. *Demographics and biomarker characteristics.*

	CN (N=7)	AD (N=21)	P-value
Sex, n (Male, %)	5 (71.4%)	10 (47.6%)	0.274
Age, y	73.4 (7.3)	69.1 (8.7)	0.246
MMSE score	28.9 (1.35)	23.0 (2.9)	<0.0001****
$A\beta_{42/40}$ ratio	0.086 (0.008)	0.041 (0.009)	<0.0001****
P-tau$_{181,}$ ng/L	27.8 (11.2)	123.0 (75.6)	0.0030**
T-tau, ng/L	291 (86)	783 (449)	0.0085**
NFL, ng/L	1112 (592)	1702 (820)	0.092
AP2B1, fmol/μL	0.59 (0.30)	0.96 (0.4)	0.057
γ-synuclein, fmol/μL	0.41 (0.15)	0.61 (0.19)	0.022*
Neurogranin, fmol/μL	0.027 (0.019)	0.060 (0.032)	0.017*
NPTXR, fmol/μL	2.32 (1.36)	2.93 (1.11)	0.247
NPTX1, fmol/μL	1.36 (1.11)	1.72 (0.80)	0.357
NPTX2, fmol/μL	0.72 (0.37)	0.94 (0.33)	0.162
GDI-1, fmol/μL	0.17 (0.063)	0.28 (0.089)	0.0059**
PEBP-1, fmol/μL	14.9 (6.3)	24.4 (7.2)	0.0047**
14-3-3 ζ/δ, fmol/μL	1.24 (0.60)	2.26 (0.69)	0.0018**
Syntaxin-1B, fmol/μL	0.10 (0.060)	0.18 (0.079)	0.018*
Syntaxin-7, fmol/μL	0.019 (0.007)	0.025 (0.008)	0.089

Notes: Data presented as mean (standard deviation). Analysis of variance (ANOVA) and chi-square goodness of fit test were used to compare continuous and categorical variables between groups. P-values: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 and **** P ≤ 0.0001. Abbreviations: AP2B1, adaptor-related protein complex 2 subunit beta 1; AD, Alzheimer's disease; $A\beta_{42/40}$, Amyloid-β 42/40; CN, Cognitively Normal; MMSE, Mini-Mental State Exam; NFL, neurofilament light; NPTX, neuronal pentraxin; PEBP-1, phosphatidylethanolamine-binding protein 1; P-tau$_{181}$, tau phosphorylated at Thr181; GDI-1, rab GDP dissociation inhibitor alpha; T-tau, total tau.