Quantification of the trans-synaptic partners neurexin-neuroligin in CSF of neurodegenerative diseases by parallel reaction monitoring mass spectrometry

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Summary

Background Synaptic proteins are increasingly studied as biomarkers for synaptic dysfunction and loss, which are early and central events in Alzheimer's disease (AD) and strongly correlate with the degree of cognitive decline. In this study, we specifically investigated the synaptic binding partners neurexin (NRXN) and neuroligin (Nlgn) proteins, to assess their biomarker's potential.

Methods we developed a parallel reaction monitoring mass spectrometric method for the simultaneous quantification of NRXNs and Nlgns in cerebrospinal fluid (CSF) of neurodegenerative diseases, focusing on AD. Specifically, NRXN-1α, NRXN-1β, NRXN-2α, NRXN-3α and Nlgn1, Nlgn2, Nlgn3 and Nlgn4 proteins were targeted.

Findings The proteins were investigated in a clinical cohort including CSF from controls (n=22), mild cognitive impairment (MCI) due to AD (n=44), MCI due to other conditions (n=46), AD (n=77) and a group of non-AD dementia (n=28). No difference in levels of NRXNs and Nlgns was found between AD (both at dementia and MCI stages) or controls or the non-AD dementia group for any of the targeted proteins. NRXN and Nlgn proteins correlated strongly with each other, but only a weak correlation with the AD core biomarkers and the synaptic biomarkers neurogranin and growth-associated protein 43, was found, possibly reflecting different pathogenic processing at the synapse.

Interpretation we conclude that NRXN and Nlgn proteins do not represent suitable biomarkers for synaptic pathology in AD. The panel developed here could aid in future investigations of the potential involvement of NRXNs and Nlgns in synaptic dysfunction in other disorders of the central nervous system.

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Keywords: synaptic proteins; neurexins; neuroligins; Alzheimer's disease; biomarkers; mass spectrometry

Abbreviations: ABC, Ammonium bicarbonate; Aβ, amyloid-beta; APP, amyloid precursor protein; Aβo, Aβ oligomers; $Aβ_{1-42}$, Aβ peptide 1-42; AD, Alzheimer's disease; CNS, central nervous system; CV, coefficient of variation; DLB, dementia with Lewy bodies; ELISA, enzyme-linked immunosorbent assays; F/T, freeze/thaw; bvFTD, behavioral variant frontotemporal dementia; GAP43, growth-associated protein 43; IS, internal standard; MRI, magnetic resonance imaging; MS, mass spectrometry; MCI, mild cognitive impairment; MMSE, mini-mental state examination; NRXNs, neurexins; NFTs, neurofibrillary tangles; Nlgns, neuroligins; PRM, parallel reaction monitoring; *p*-tau, phospho-tau; QC, quality control; SPE, solid-phase extraction; *t*-tau, total-tau; VaD, vascular dementia

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Research in context

Evidence before this study

The fluid biomarker field for neurodegenerative diseases is rapidly expanding and many investigations are directed towards the study of synaptic proteins as biomarkers for synaptic dysfunction and loss. Alterations of synaptic integrity is an early and central event in Alzheimer's disease (AD) and other neurodegenerative diseases, which also correlate with the degree of cognitive decline. Therefore, synaptic biomarkers are sought after as they might hold the ability of detecting early pathological changes and to follow disease progression. Neurexin (NRXN) and neuroligin (Nlgn) proteins are binding partners ubiquitously expressed at neuronal synapses, binding to each other at the synaptic cleft through their extracellular domains. NRXNs have been detected in cerebrospinal fluid (CSF), where several studies have reported altered levels of the proteins in AD, already at the mild cognitive impairment (MCI) stage, albeit with variable results. In our previous work, we investigated Nlgn1 in brain and CSF of AD patients, with promising results, but the other members of the Nlgn's family have never been explored as biomarkers. None of the previous studies investigated these proteins simultaneously. Given the importance of NRXNs and Nlgns at the synapse and the tight relationship these two families of proteins show, we decided with this study to investigate them simultaneously in a comprehensive clinical cohort, focusing on AD, but also including MCI patients, both due to AD and to other causes, and a non-AD dementia group.

Added value of this study

To our knowledge, this is the first study that simultaneously investigated NRXNs and Nlgns in CSF of AD patients. Through their extracellular domains, the presynaptic NRXNs bind the postsynaptic Nlgns across the synaptic cleft and their mechanisms appear to be interrelated. This study tried to answer the question whether these proteins change in AD patients and whether changes in the levels of NRXN proteins would affect Nlgns and vice versa. Moreover, the method developed here allows for their simultaneous quantification with high sensitivity and precision. In order to create this mass spectrometry panel, several different peptides per protein have been included, covering the extracellular part of NRXN and Nlgn proteins, of which soluble fragments are quantifiable in CSF. The study aims at reproducing previously published results and expand them, in order to increase our knowledge on NRXN and Nlgn proteins as possible synaptic biomarkers for AD.

Implications of all the available evidence

Despite previous studies showing NRXN proteins changing in CSF of AD patients, even at early stages, our results show no changes of these proteins in both AD dementia and MCI cases, as well as in the non-AD dementia group. Nlgns also do not show changes in their levels in any of the groups analysed. Thus, this study does not support the use of NRXNs and Nlgns as biomarkers for synaptic dysfunction in AD. If changes of these proteins happen at even earlier stages than those we investigated, or in different brain regions, or in other brain pathologies, are questions that remain to be investigated. Moreover, these results might suggest that synaptic dysfunction in AD is not a generalized disruption of synapses but it could entail more specific mechanisms, which affect a subset of proteins but not others. The work presented in this paper provides a novel methodology, which can be used for further specific studies of these proteins in order to elucidate their role at the synapse and implications in neurodegenerative and neuropsychiatric diseases.

Introduction

Synapses are central structures for memory function and information storage in the brain, and as such, their integrity and homeostasis are essential for proper cognitive function. Synapse health is disrupted in Alzheimer's disease (AD), the most common cause of dementia, where abnormal depositions of proteins or peptides, such as amyloid-beta (Aβ) in plaques and tau protein in neurofibrillary tangles (NFTs), leads to synaptic degeneration, neuronal loss and clinical symptoms.[1-4] Additionally, synaptic dysfunction and synapse loss have also been shown in other types of dementia.[5-9] However, the mechanisms leading to synaptic loss are not fully understood yet and further investigations are needed. Considering the high correlation between synaptic loss and cognitive symptom severity in AD,[10,11] the study of synaptic proteins could increase our understanding of the pathophysiological processes underlying neurodegenerative changes and possibly yield valuable biomarkers to monitor them. The potential usefulness of synaptic biomarkers is also founded on the fact that synapses are the substrate of cognition and synaptic dysfunction is one of the earliest events in the course of neurodegenerative diseases. Therefore, it is suggested that pathological processing of synaptic proteins may reflect changes in cognition in neurodegenerative diseases at early stages.[3,12-14] In AD, the Aβ peptide 1-42 (A$\beta_{1\text{-}42}$) or the A$\beta_{42/40}$ ratio, total-tau (*t*-tau) and tau phosphorylated at Thr181 (*p*-tau$_{181}$) are well-established cerebrospinal fluid (CSF) biomarkers[15] describing the main pathological events in the brain during the course of the disease. Several synaptic proteins have also been investigated as biomarkers.[16] Among them neurogranin, growth-associated protein 43 (GAP43), synaptosomal-associated protein 25 (SNAP25) and synaptotagmin-1 are arguably the most investigated in AD.[17,18] However, the complexity of the brain and the multiple mechanisms involved in synaptic regulation call for more investigations into processing of synaptic proteins to better understand different events in disease

progression and to better discriminate between different dementias.

Correct interaction between the pre- and post-synaptic compartment is essential for proper function of synapses. Neurexin (NRXN) and neuroligin (Nlgn) families consist of important synaptic adhesion proteins taking part in this fundamental process.[19-21] The presynaptic NRXNs and postsynaptic Nlgns[22] comprise single-pass transmembrane proteins with short cytoplasmic domains and large extracellular domains, through which they bind each other in the synaptic cleft, stabilizing the two compartments of the synaptic bouton.[20] Moreover, NRXNs and Nlgns cluster receptors and channels essential for synapse formation and differentiation of the synaptic compartment. How the different proteins exert their function is not entirely understood. However, NRXNs and Nlgns appear to have a tight relationship by which they regulate each other and downstream signalling through their interaction. To exert their function, both their extracellular and cytoplasmic domains seem to be important.[19,23] NRXNs in humans are encoded by three genes which use two different promoters. Their transcription gives rise to α- and β-forms of NRXNs.[19,24] NRXNs contain relatively well conserved cytoplasmic domains, but differ much more in their extracellular domain, where they can be spliced at five alternative positions, and theoretically produce more than a thousand different isoforms of the proteins.[25] In humans, five *NLGN* genes have been described. Nlgn1, -2 and -3 proteins are predominantly expressed in the central nervous system (CNS) and appear to be the most abundant.[24] Nlgn4 is designated as Nlgn4-X to distinguish it from the product of the fifth gene, Nlgn4-Y (occasionally referred to as Nlgn5), which is located on the Y chromosome and presents high sequence homology to Nlgn4-X.[26] Although it is the least studied of the family, also Nlgn4 has been found expressed in the cerebral cortex, preferentially localized at dendritic spines, taking part in excitatory synaptic transmission.[27]

Genetic alterations of these proteins have been connected to synaptic dysfunction in mental disorders like schizophrenia and autism,[25,28,29] and various studies have reported NRXNs and Nlgns to be altered in neurodegenerative diseases, like AD.[30-34] Nlgn1 protein levels has been shown to be decreased in different brain regions of AD and a group of primary tauopathies.[18,35] Moreover, the extracellular domains of NRXNs and Nlgns undergo proteolytic cleavage[36-39] which leads to the extracellular release of a soluble N-terminal ectodomain that can be detected in CSF. Independent studies have reported altered levels of NRXN-1α,[40-43] NRXN-2α, NRXN-3α and Nlgn2 in CSF of AD[41,44,45] and from the earlier stage of mild cognitive impairment (MCI).[46] However, these studies showed variable results and none of them investigated NRXNs and Nlgns simultaneously. Considering these investigations in brain and CSF and the tight relationship between NRXNs and Nlgns, the simultaneous monitoring of these proteins and the possibility to distinguish between the different isoforms would be highly valuable to increase our knowledge of the processes regulating these proteins at the synapse and evaluate the potential of using these proteins as biomarkers for synapse dysfunction in pathology. The heterogeneous nature of NRXN and Nlgn proteins complicates the use of antibody-based methods, whereas high-throughput and highly selective methods such as mass spectrometry (MS), offers an antibody-independent alternative, as well as the possibility of multiplexing. Indeed, MS is increasingly used to investigate biomarkers, showing good performance and reproducibility.[47] Therefore, the aim of this study was to develop a targeted MS assay for the simultaneous quantification of NRXN and Nlgn proteins to study these proteins in CSF samples from patients of neurodegenerative diseases. Included in the parallel reaction monitoring (PRM) assay are the most commonly explored NRXN-2α, -3α, -1α and -1β and all the Nlgn proteins, Nlgn1 to 4. In a pilot study, we used the novel assay to explore the proteins in CSF having an AD and non-AD profile (defined by biochemical criteria). Subsequently, the study of the proteins was expanded to a clinical cohort including CSF from controls, AD at both MCI and dementia stages, and a group of non-AD MCI and non-AD dementia individuals.

Methods

CSF samples included in the study

Discovery cohort. The NRXNs-Nlgns MS panel was initially tested in a pilot study of CSF samples from the Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden, including AD CSF profile (n=21) and non-AD CSF profile (n=19). These samples were biochemically defined as AD and non-AD samples based on the analysis of CSF core biomarkers for AD (p-tau$_{181}$, t-tau and Aβ_{1-42}) following the respective diagnostic criteria.[48]

Clinical cohort. All included subjects were recruited at the Cognitive Neurology Centre, GHU APHP Nord Université de Paris Lariboisière Fernand Widal Paris, from 2015 to 2019. All methods and diagnosis processes have been extensively described in Tible *et al.*[17] Briefly, recruited individuals were seen at the memory clinic and underwent CSF biomarkers analysis for a cognitive complaint. Consensus clinical diagnosis of the neurocognitive disorders was reached after review by a multidisciplinary team according to validated diagnostic criteria. Reference criteria were used for the inclusions of patients including those for AD dementia,[49] MCI due to AD (MCI-AD),[50] and for patients with other

dementia (non-AD dementia) including vascular dementia (VaD),[51] dementia with Lewy bodies (DLB),[52] and frontotemporal dementia of behavioural variant type (bvFTD).[53] MCI of non-neurodegenerative causes (non-AD MCI) were also included. The neurological control (NC) group included participants with subjective cognitive complain or non-neurological disorders. CSF core biomarker analysis was performed (see below) and, after analysis, AD and MCI due to AD had pathological amyloid ratio, high *p*-tau and *t*-tau, while neurological control subjects had normal CSF profile. Other dementia and neurological control groups had a CSF amyloid ratio in the range of normal.

CSF sampling and analysis of AD core biomarkers

CSF samples were obtained by lumbar puncture according to the European and French recommendations[54,55] in the context of the diagnostic workup of patients with cognitive complaint or decline. CSF was collected in polypropylene tubes using a standardized procedure. Samples were centrifuged (2000 × g, 20 min, +4°C) and the supernatant stored at -80°C. For the discovery cohort, CSF AD core biomarker concentrations were quantified by commercially available INNOTEST enzyme-linked immunosorbent assay (ELISA) ($A\beta_{1\text{-}42}$ cat.# 81583, *t*-tau cat.# 81572, *p*-tau_{181} cat.# 81581; Fujirebio, Ghent, Belgium). For analysis of CSF core AD biomarkers in the clinical cohort, a LUMIPULSE G1200 instrument (Fujirebio, Ghent, Belgium) was used according to the manufacturer's instructions. The normal ranges were defined as follows: $A\beta_{42}$ > 620 ng/L, $A\beta_{42/40}$ ratio > 0.61, *p*-tau_{181} < 61 ng/L, *t*-tau < 479 ng/L.[56] Analysis of the synaptic biomarkers neurogranin and GAP43 was performed using in-house ELISA assays[57,58] at the Neurochemistry Laboratory at Sahlgrenska University Hospital, Mölndal, Sweden. Quality controls (QC) used for assay validation consisted of pooled CSF samples, also obtained from the Neurochemistry Laboratory, which were aliquoted and stored in the same way as the individual patient samples. Demographics and biomarker characteristics of the patients are shown in Table 1.

Ethics

For the discovery cohort, the collection and storage of CSF samples were in accordance with the Swedish law of biobanks in healthcare (2002:297). The use of these patient samples was approved by the Ethics Committee at the University of Gothenburg (EPN 140811). For the clinical cohort, the study was approved by the Bichat Hospital Ethics Committee of Paris Diderot University. All patients signed an informed consent.

Peptide selection

For each protein, peptides were chosen either based on previously published studies[18,40,44] or selected based on sequence uniqueness, length, and amino acid composition after *in silico* tryptic digestion (Table 2). Exceptions to the uniqueness criteria were; peptide 3 for NRXN-1 (VDSSSGLGDYLELHIHQGK), which is common to the two forms NRXN-1α and NRXN-1β, and all peptides for Nlgn4, which are found in both Nlgn4-X and Nlgn4-Y. All the peptides belong to the extracellular domain of the proteins, which is released extracellularly after proteolytic processing.

Heavy-isotope-labelled standards

Thirty-one tryptic peptides, labelled at the C-terminal arginine or lysine with ^{13}C and ^{15}N, were purchased from JPT Peptide Technology (Berlin, Germany) and used as heavy-isotope-labelled internal standards (IS) for peptide quantification. Peptides (stated amount from the manufacturer ≈ 10 nmol) were reconstituted in 1 mL 10% acetonitrile solution in deionized water (v/v), aliquoted and stored at -20°C pending analysis. Aliquots of all peptides were pooled and diluted in 50 mM ammonium bicarbonate (ABC) to a final optimized concentration, in order to create an IS mix matching the respective protein levels in the CSF samples.

Sample preparation and SPE

For sample preparation, 100 µL of CSF samples were pipetted into Micronic 0.75-mL tubes (cat.# MP32069L), followed by the addition of 25 µL IS diluted in 50 mM ABC as mentioned above. Cysteine disulfide bridges in the samples were reduced by adding 25 µL of 30 mM dithiothreitol in 50 mM ABC and shaken for 30 min at 60°C. Samples were then cooled to room temperature and cysteines were blocked by alkylation with 25 µL 70 mM iodoacetamide in 50 mM ABC and shaken for 30 min in the dark. Next, 25 µL of trypsin/Lys-C (Promega, cat.# V5073), corresponding to 0.5 µg per sample, were added to every sample and digestion was performed at 37°C overnight (16 h) shaking at 90 rpm. The day after, samples were spun down, centrifuged and digestion was stopped by the addition of 25 µL 10% trifluoroacetic acid in deionized water (v/v). Subsequently, solid-phase extraction for sample clean-up from salt and detergents was performed using Oasis 30 μm HLB 96-well μElution Plates (Waters Co., Milford, MA, USA). The plates were first conditioned twice with 300 µL of methanol, then equilibrated twice with 300 µL deionized water using a rotary pump for controlled suction. Samples were then loaded into the plate wells, aspirated and washed twice with 300 µL of deionized water. Finally, elution of the samples was obtained by the addition of 2 × 100 µL of methanol, and eluates collected into Micronic 0.75-mL tubes. Subsequently, eluates were dried in a vacuum centrifuge and stored at -80°C pending analysis. For the analysis of the clinical cohort, samples were randomized in three 96-well plates, and eight QC samples (two different

Patient group	Discovery cohort		Clinical cohort from Paris				
	Non-AD (n=19)	AD (n=21)	NC (n=22)	MCI-AD (n=44)	AD dementia (n=77)	Non-AD MCI (n=46)	Non-AD Dementia (n=28)
Sex (male/female)	7/11[a]	12/9	8/14	17/27	28/49	17/29	16/12
Age (years) mean, [SD]	54.2 [8.7]	72.4 [9.7]	64.4 [8.86]	72 [7.7][*&]	72.2 [8.33][*&]	67 [10][#]	66 [7.5][#]
MMSE, mean, [SD]	-	-	27.14 [2.34]	23.34[4.54][*#]	19.06 [5.63][*]	24.5 [3.65][#]	23.5 [4.95][#]
Biomarkers							
Aβ_{42} [pg/ml] mean, [SD]	891 [173.32]	346.9 [86.26]	1095.9 [277.09]	599 [332.04][*&]	508.33 [160.9][*&]	1080.5 [419.8][#]	974.9 [380.9][#]
Aβ_{42}/Aβ_{40} mean, [SD]	-	-	0.093 [0.009]	0.046 [0.012][*&]	0.042 [0.009][*&]	0.090 [0.010][#]	0.089 [0.014][#]
t-Tau [pg/ml] mean, [SD]	227.6 [65.56]	530.19 [46.60]	243.09 [66.09]	594.63 [268.8][*&]	731.79 [384.9][*&]	296.35 [139.6][#]	329.1 [303.7][#]
p-Tau [pg/ml] mean, [SD]	37.28 [8.95]	67.43 [3.89]	32.84 [8.06]	92.42 [46.06][*#&]	112.59 [55.86][*&]	37.07 [15.41][#]	34 [10.63][#]
Neurogranin [pg/ml] mean, [SD]	-	-	131.69 [40.19]	259.8 [82.72][*&]	268.72 [78.61][*&]	172.4 [45.11][#]	163.3 [49.47][#]
GAP43 [pg/ml] mean, [SD]	-	-	2427.26 [707.16]	4071.3 [1889.2][*&]	4497 [1828.5][*]	3071.58 [1263.7][#]	3012.9 [1422.6][#]

Table 1: **Demographic characteristics, CSF AD core biomarkers and synaptic biomarkers concentration for the discovery and the validation cohorts**

Abbreviations: AD= Alzheimer's disease, NC=neurological controls, MCI=mild cognitive impairment, GAP43=growth-associated protein 43, MMSE=Mini-Mental State Examination, SD=standard deviation

[a] One missing dataNotes: Analysis of variance followed by Tukey's post hoc test (continuous variables) or contingency chi-square test (sex), were used to test differences between the groups. Significant differences compared to control (*) or AD (#) or non-AD dementia (&) in the same cohort are highlighted. Significance level set to $p \leq 0.05$.I will add the difference between the groups in the Paris cohort

QCs in four replicates per plate) were added to account for variations and to allow performance check during MS analysis. Median of the total area ratio of QC1 was used for adjustment of plate variations.

PRM-MS and data analysis

The PRM-MS analysis was performed using a Q Exactive hybrid quadrupole-orbitrap high resolution mass spectrometer (Thermo Fisher Scientific), with electrospray ionization. On the day of analysis, samples were reconstituted in 100 µL of 50 mM ABC, shaken for 30 min at room temperature and 45 µL were loaded using a Vanquish UHPLC (Thermo Fisher Scientific). Sample peptides were separated on a Hypersil Gold reversed-phase column (particle size 1.9 μm, internal diameter 2.1 mm, length 100 mm, Thermo Fisher Scientific) operated at a flow rate of 300 μL/min by applying a broken gradient of 0-32% B for 24 min (total sample cycle time was 32 min). Mobile phases used were A: 0.1% formic acid in deionized water (v/v) and B: 0.1% formic acid and 84% acetonitrile in deionized water (v/v/v). A graphical representation of the gradient is shown in Supplementary Fig. 1. Electrospray conditions were set as follows; spray voltage at +4100 V, capillary temperature at 320°C, sheath gas setting of 25, aux gas setting of 10, sweep gas setting of 0, probe heater temperature at 300°C, and S-lens RF level setting of 55. Mass spectra were acquired using a scheduled PRM method with retention time windows of 2 min for each peptide and a toggle limit of four different peptide pairs. For data acquisition, isolation window was set to 3 m/z units, automatic gain control target value to 3×10^6 and maximum injection time to 250 ms with a matching resolution setting of 70,000.

For each peptide, collision energy was optimized in order to maximize the sensitivity of the PRM assay. Peak detection and area integration was performed using Skyline 20.2 (MacCoss Lab Software).[59] Every peak was manually inspected and, when required, peak adjustment and removal of transitions affected by interference were applied. Relative peptide quantification was performed by dividing the sum of all measured fragment peak areas by the sum of the fragment peak areas of the corresponding IS. Representative peaks and corresponding transitions are shown in Supplementary Fig. 2.

Assay validation

To monitor intra- and inter-plate variations, peptide stability and method and dilution linearity, CSF pools were used as QC standards for the different tests. The variability was estimated by calculating the coefficient of variation (CV) for the QCs. In order to investigate the stability of NRXN and Nlgn peptides, different storage conditions and freeze/thaw (F/T) cycles were tested. Aliquots of CSF samples from six different individuals

Protein name	Protein abbreviation	Protein accession ID	Peptide sequence	Position
Neurexin-1α (3 peptides)	NRXN-1α	Q9ULB1	EATVLSYDGSMFMK	[714-727]
			LTVDDQQAMTGQMAGDHTR	[822-840]
			VDSSSGLGDYLELHIHQGK [c]	[1167-1185]
Neurexin-2α (4 peptides)	NRXN-2α	Q9P2S2	TALAVDGEAR	[123-132]
			LSALTLSTVK	[160-169]
			LGERPPALLGSQGLR	[183-197]
			LQGDLSFR	[477-484]
Neurexin-3α (4 peptides)	NRXN-3α	Q9Y4C0	SDLSFQFK	[48-55]
			NGLILHTGK	[292-300]
			ANDGEWYHVDIQR	[536-548]
			FICDCTGTGYWGR	[664-676]
Neuroligin-1 (3 peptides)	Nlgn1	Q8N2Q7	LDDVDPLVATNFGK	[47-60]
			WTSENIGFFGGDPLR	[279-293]
			FEEVAWTR	[609-616]
Neuroligin-2 (3 peptides)	Nlgn2	Q8NFZ4	FQPPEAPASWPGVR	[83-96]
			ELVDQDVQPAR	[335-345]
			TLLALFTDHQWVAPAVATAK	[449-468]
Neuroligin-3 (1 peptide)	Nlgn3	Q9NZ94	VGCNVLDTVDMVDCLR	[337-352]
Neuroligin-4 (2 peptides)	Nlgn4	Q8N0W4/	WIEENVGAFGGDPK [d]	[232-245]
		Q8NFZ3	TGPEDTTVLIETK [d]	[655-667]

***Table 2*: Proteins and peptides included in the PRM-MS study**
[a]Underlined cysteine (C) indicate the carbamidomethylation occuring through alkylation
[b]Blue colour-coded amino acids indicate heavy labelled [U-^{13}C6,^{15}N4]-arginine (R) and [U-^{13}C6,^{15}N2]-lysine (K)
[c] Common peptide for NRXN-1α and NRXN-1β
[d] Common peptides for the two Nlgn4 isoforms

under different storage conditions and F/T cycles were analysed. F/T cycles were as follows: aliquot #1 was stored at -80°C (*i.e.*, one F/T cycle), while aliquots #2, #3, #4 and #5 underwent a total of two, three, four and five F/T cycles (and stored at -80°C in between cycles), respectively. Storage conditions were as follows: aliquot #6 at 5-8°C for 24 h and then stored at -80°C, aliquot #7 at 5-8°C for one week and then stored at -80°C, aliquot #8 at room temperature for 24 h and then stored at -80° C, aliquot #9 stored at -20°C for one month, then at -80°C. To evaluate the linearity of the method, four-fold serial dilutions of the IS were prepared and spiked into QC samples, digested and analysed in triplicate by LC-MS/MS. To examine the possible matrix effects caused by the CSF amount, dilution linearity of the peptides was tested using different volumes of CSF; volumes tested were 120, 100, 80, 60 and 40 µL of QC samples. For both tests, the curve fits were created using weighted sum of squares ($1/X^2$) to account for non-homogeneous distribution of the residuals.[60]

Statistical analysis

Data related to NRXNs and Nlgns were not normally distributed (even after logarithmic transformation) and, therefore, non-parametric tests were applied. Differences between groups were assessed using Mann-Whitney U test or Kruskal-Wallis test with Dunn's correction for multiple comparisons, when appropriate. Correlations were investigated using Spearman's rank correlation coefficient (rho). None of the peptides correlated with age and sex, therefore neither age nor sex were included as covariates, except for Nlgn1 and Nlgn4. For Nlgn1 data were adjusted for age and for Nlgn4 data were adjusted for age and sex (Supplementary table 1). The Nlgn1 and Nlgn4 data were log transformed to make it resemble a normal distribution, thus linear regression of analysis of covariance was used. The cut-off for Aβ +/Aβ- was defined by the $A\beta_{42/40}$ ratio. For data normally distributed, differences between more than two groups were investigated using analysis of variance followed by Tukey's post hoc test (continuous variables) or contingency chi-square test (categorical variable). Data visualization and statistical analysis were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). All tests were two-sided and p-values ≤ 0.05 were considered as the threshold level for significance. However, the p-values for correlations were adjusted using Bonferroni correction for multiple comparison (n=24) and consequently a probability of $p \leq 0.002$ was considered statistically significant.

Role of funders

A declaration of interest section, where funders are listed, can be found at the end of the manuscript.

Funders did not have any role in the study design, data collection or analysis and interpretation of the results or manuscript writing. All authors had complete access to the data.

Results

Assay validation and performance

In total, 31 tryptic NRXN and Nlgn peptides were investigated in the CSF samples. Based on the repeatability analysis performed using eight QC replicates, 11 peptides could not be quantified (CSF level below limit of detection) or showed a CV higher than 20% (CSF level below limit of quantification); they were therefore disregarded from further analysis (excluded peptides are highlighted in Supplementary Table 2). Further, after peptide analysis and quantification in the clinical cohort, an additional four peptides were not taken into account for group comparison for equivalent reasons (highlighted in Supplementary Table 3). In total, 16 peptides (ten for NRXNs and six for Nlgns) were utilized for group comparison and final analysis in the clinical cohort (acquisition characteristics and repeatability analysis of the selected peptides are shown in Supplementary Table 3). The NRXN-1 peptide 3 sequence is common for both NRXN-1α and NRXN-1β proteins. However, from the initial screening, the unique peptide for NRXN-1β showed high variability, probably because of very low abundance of the protein in CSF. Indeed, α-forms of neurexins have been shown to be more abundant than the corresponding β-forms.[61] Taking these pieces of evidence together, we believe NRXN-1 peptide 3 mainly reflects NRXN-1α. All the selected peptides for NRXNs and Nlgns showed analytical stability when the CSF samples underwent up to five F/T cycles, except for Nlgn2 peptide 1, Nlgn4 peptide 1, and NRXN-3α peptides 3 and 4. The same peptides showed high variation also upon different storage conditions, whereas the others were found to be stable (Supplementary Fig. 3). The method linearity test and the dilution linearity test, showed that the relative error corresponding to the back-calculated concentration were within the set limit (20%) for most of the peptides and at least for one peptide per protein (Supplementary Fig. 4 and 5). As mentioned above, several peptides showed suboptimal performance, especially among Nlgns, and were thus discarded from further analysis.

NRXNs-Nlgns CSF levels

The NRXNs-Nlgns panel was initially evaluated in a pilot cohort, which included CSF samples from 21 AD and 19 non-AD patients. All the selected peptides were quantified, but no significant differences were found between the groups (Fig. 1). In order to extend these preliminary observations and further investigate the NRXN and Nlgn proteins in the AD continuum and in other neurodegenerative disorders, a larger clinical cohort including CSF from neurological controls (NC, n=22), AD (n=77), MCI due to AD (n=44), non-AD MCI (n=46) and non-AD dementia (n=28) was investigated. The CSF concentration and QC CV of all NRXN and Nlgn peptides are shown in Supplementary Table 3. Neither NRXNs nor Nlgns showed any significant change in AD, or in the MCI due to AD group when compared with neurological controls. Protein levels also did not change in the non-AD dementia and the non-AD related MCI groups, when compared with neurological controls or AD (Fig. 2). For a more comprehensive investigation, the non-AD dementia group was further divided into its constitutive subgroups, VaD (n=3), DLB (n=12), and bvFTD (n=13), and group differences in NRXNs and Nlgns levels were investigated. The proteins did not show any difference when the subgroups were compared to neurological controls (Supplementary Fig. 6). Protein levels were further investigated in relation to Aβ pathology, and groups were dichotomized into Aβ+ and Aβ- based on the A$\beta_{42/40}$ ratio. Neither NRXNs nor Nlgns levels differed between the two groups, except for Nlgn2 (p=0.024, Mann-Whitney U test) although the difference was not deemed of importance because of the high overlap between the groups (Fig. 3). Nlgn1 peptide showed a significant correlation with age, and Nlgn4 peptide two, with age and sex (Supplementary Table 1), but correcting for it did not change the results. NRXN peptides were highly correlated with each other (rho=0.68-0.97, p≤0.002, Spearman's rank correlation coefficient test). The same strong correlation was also seen for all Nlgn peptides (rho=0.50-0.93, p≤0.002, Spearman's rank correlation coefficient test). NRXN and Nlgn proteins also significantly correlated with each other (Supplementary Fig. 7). A moderate to strong correlation between NRXN and Nlgn peptides was, with a few exceptions, found in all the groups (neurological control group rho=0.40-0.96, AD group rho=0.48-0.97, MCI due to AD rho=0.42-0.98, non-AD MCI rho=0.62-98, non-AD dementia rho=0.34-0.96, p≤0.002, Spearman's rank correlation coefficient test). Further, there was no change in correlations between the different groups (Fig. 4). NRXN and Nlgn proteins did not correlate with A$\beta_{42/40}$ ratio (in any of the groups), whereas both correlated quite strongly with Aβ_{40} and moderately with Aβ_{42} across all groups. Generally, NRXNs and Nlgns moderately correlated also with *t*-tau and to a lesser degree with *p*-tau. When comparing NRXNs and Nlgns with other synaptic biomarkers, they correlated moderately with GAP43 but to a lesser extent with neurogranin (Fig. 4). Neurogranin and GAP43 moderately correlated with each other in the AD group (rho=0.56, p≤0.002, Spearman's rank correlation coefficient test) and in the non-AD dementia group (rho=0.76, p≤0.002, Spearman's rank correlation coefficient test), whereas a weak non-significant correlation was found in the other groups (neurological

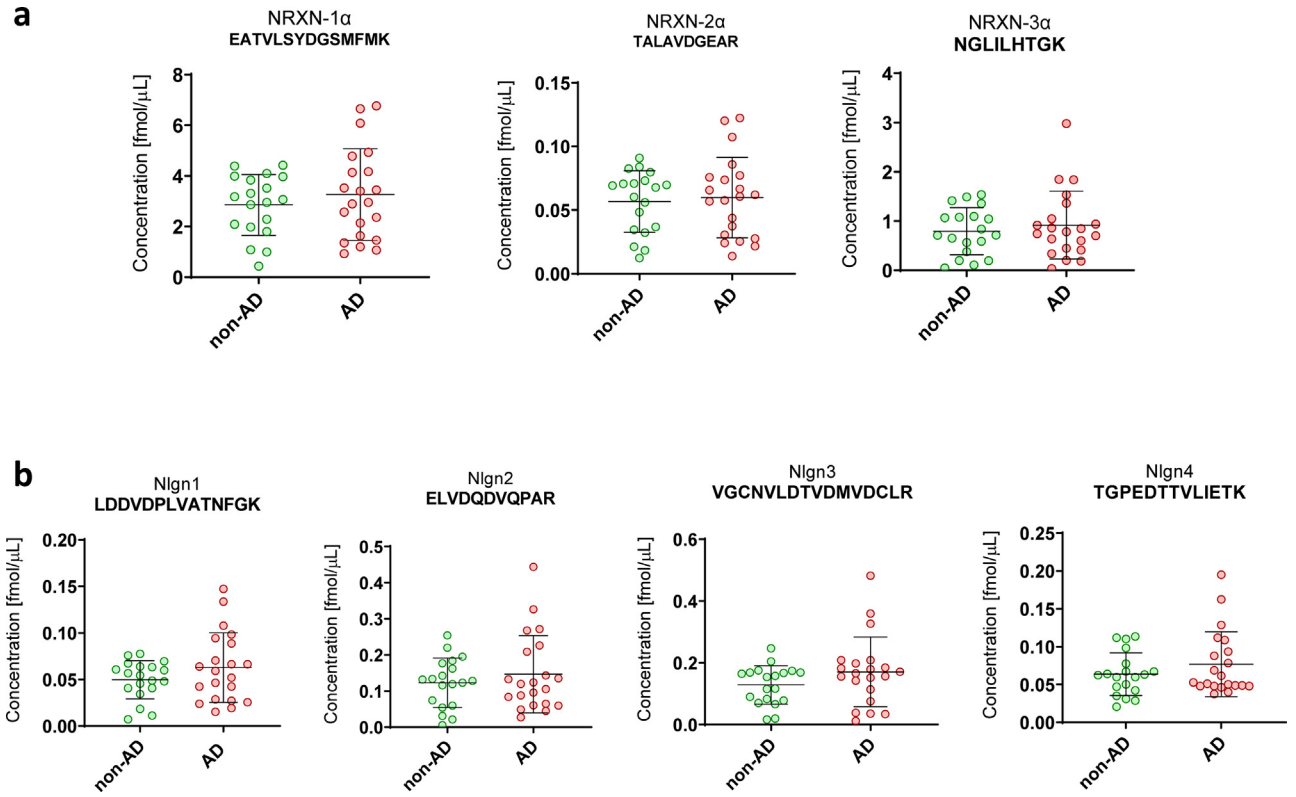

Figure 1. *CSF concentrations obtained by PRM analysis of the NRXNs-Nlgns panel in the pilot cohort.* In the figure, only one representative peptide for (**a**) NRXNs and (**b**) Nlgns is shown. The cohort consisted of biochemically defined non-Alzheimer's disease CSF profile (non-AD, n=19) and Alzheimer's disease (AD, n=21) CSF samples. Samples were analysed as singlicates. The bars indicate median with interquartile range.

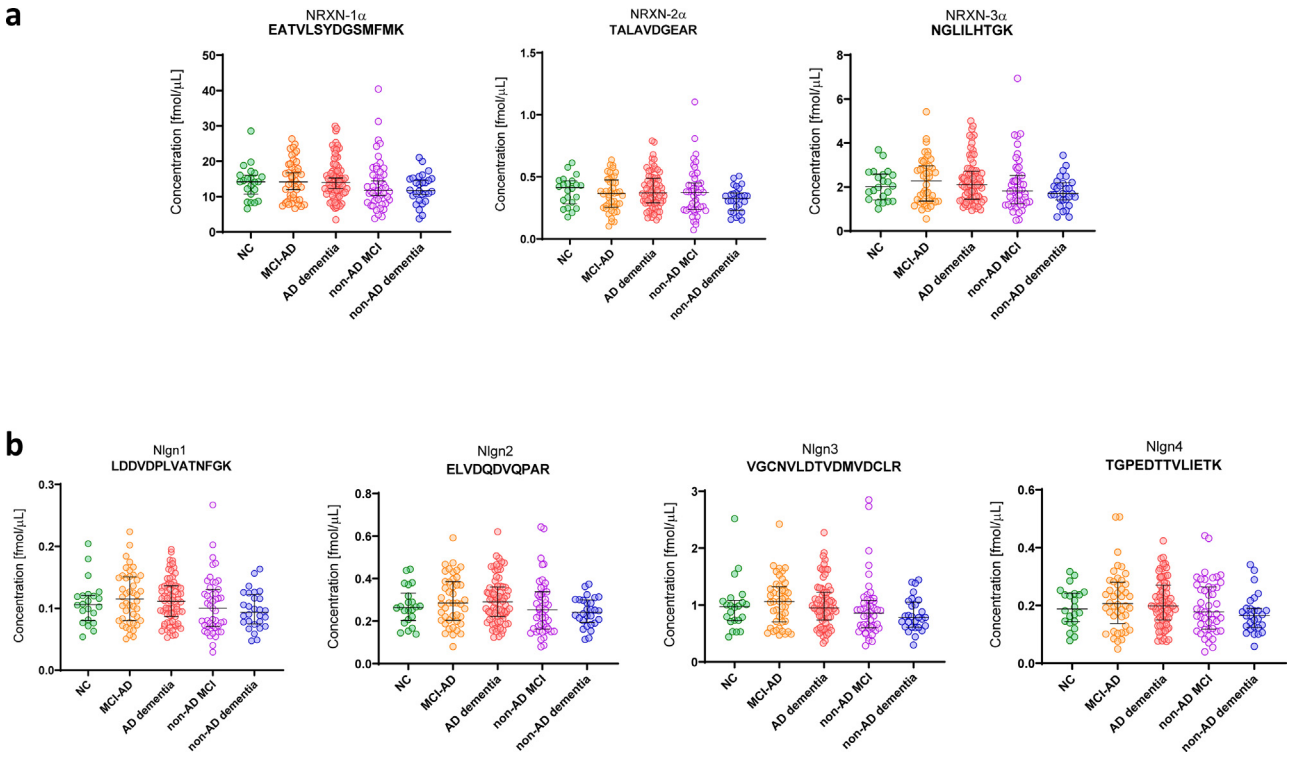

Figure 2. *CSF concentrations obtained by PRM analysis of the NRXNs-Nlgns panel in the clinical cohort.* In the figure, only one representative peptide for (**a**) NRXNs and (**b**) Nlgns is shown. The clinical cohort consisted of neurological controls (NC, n=22), Alzheimer's disease dementia (AD dementia, n=77), mild cognitive impairment due to AD (MCI-AD, n=44), MCI non-due to AD (non-AD MCI, n=46) and a group of non-AD dementia (n=28). Samples were analysed as singlicates. The bars indicate median with interquartile range.

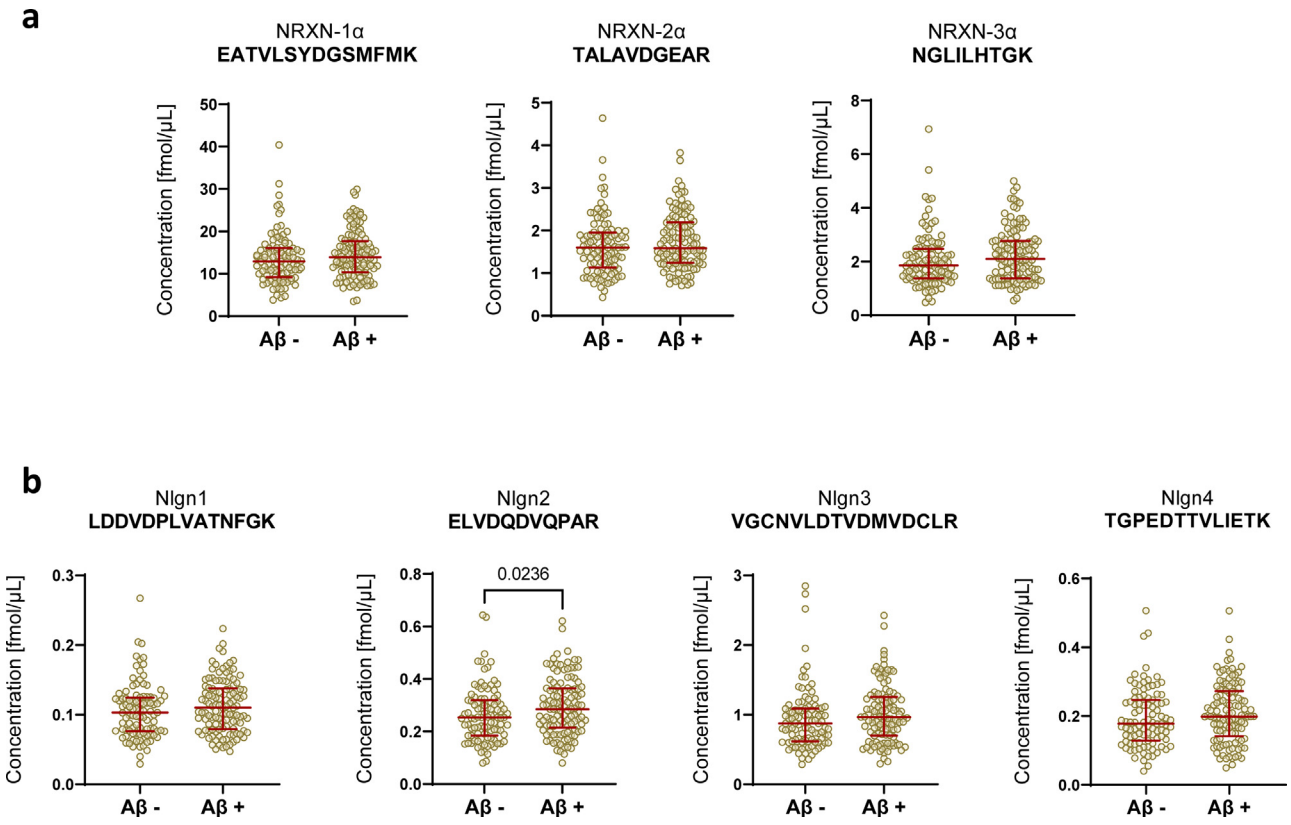

Figure 3. *NRXNs and Nlgns levels in relation to Aβ pathology.* In the figure, only one representative peptide for (**a**) NRXNs and (**b**) Nlgns is shown. Peptide levels are compared in dichotomized Aβ+ and Aβ- groups. Nlgn2 was slightly, although significantly increased, in the Aβ+ group ($p \leq 0.05$, Mann-Whitney U test). The bars indicate median with interquartile range.

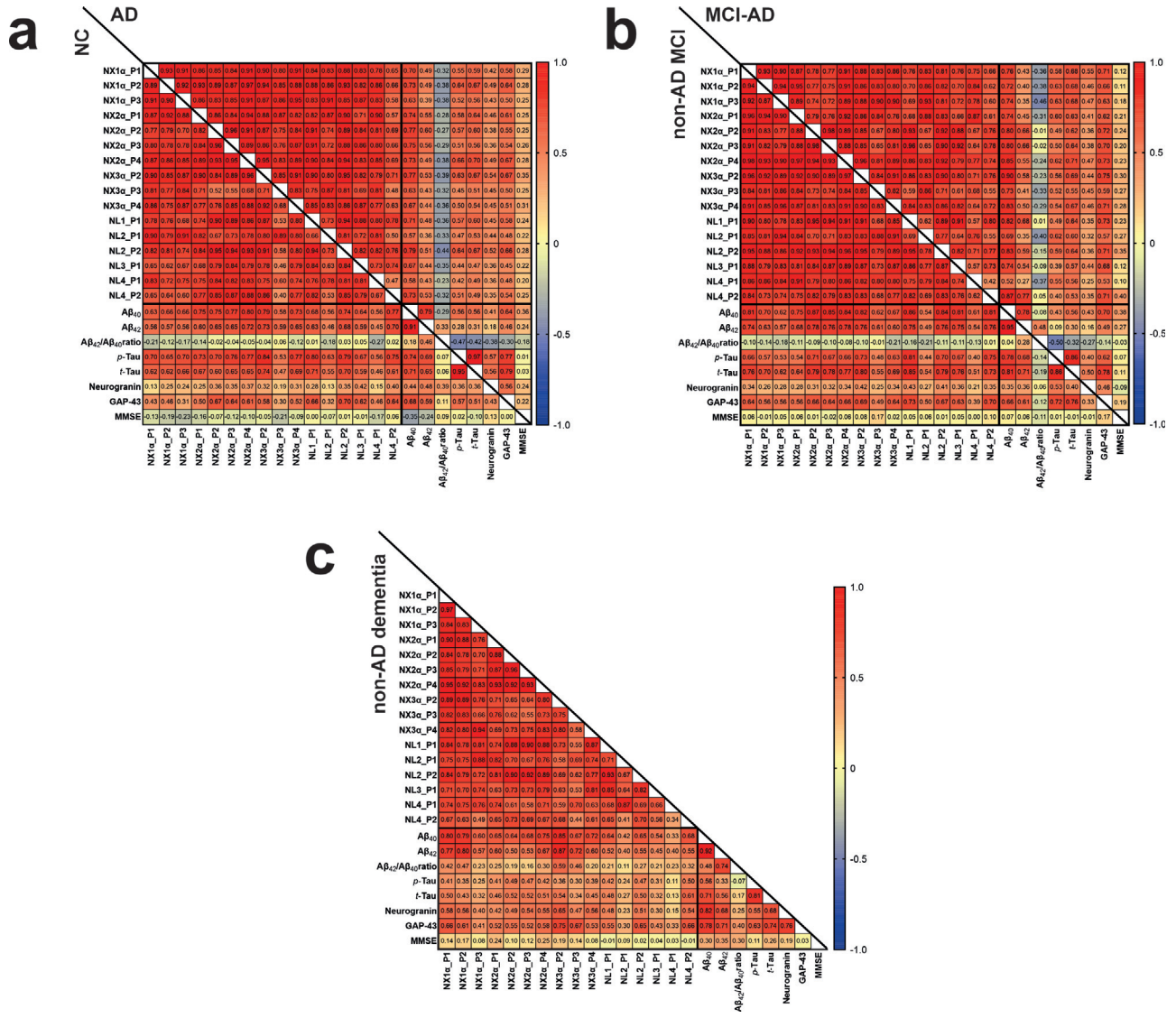

Figure 4. *Correlation matrix between CSF correlation of NRXNs and Nlgns peptides*. (**a**) Neurological controls (NC) and AD, (**b**) MCI-AD and non-AD MCI, (**c**) non-AD dementia, in the Paris clinical cohort. The correlation coefficients are presented as Spearman's rho. Abbreviations: NX=neurexins, NL=neuroligins, P=peptide number

control group rho=0.43, MCI due to AD group rho=0.46, non-AD MCI rho=0.33). Neither NRXN nor Nlgn peptides correlated with the Mini-Mental State Examination (MMSE) score (Fig. 4). The correlations of the NRXNs and Nlgns and relative p-values are summarized in Supplementary Table 4.

Discussion

In this study, we have developed a targeted MS method for the simultaneous quantification of NRXNs and Nlgns in CSF of neurodegenerative diseases. The focus of the investigation was on AD, thus the panel was tested in a discovery cohort, comprising patients showing a typical AD CSF biomarkers profile and, subsequently, in a larger clinical cohort. The results obtained in both cohorts indicate no significant change in CSF levels of NRXNs and Nlgns, neither in the AD continuum (represented by the MCI due to AD cases and AD dementia patients), nor in the non-AD MCI group, when compared with neurological controls. The non-AD dementia group, including VaD, DLB, and bvFTD cases, also did not show any statistically significant difference in the levels of NRXNs and Nlgns. In our previous study on CSF samples from AD and neurological controls using western blot for quantification,[18] we found that Nlgn1 levels showed weak or no difference between the two groups. Similarly, in the present study, the different levels of Nlgns between the AD and the neurological control groups did not reach significance. Several previous studies by other groups showed that levels of NRXNs are changed in CSF of AD or MCI patients, although in different directions. Decreased levels in CSF of AD cases have been reported for NRXN-1α and -3α but not for NRXN-2α.[40,42] NRXN-1α, -2α and -3α were found to be elevated in MCI, with NRXN-3α showing the best performance in separating the groups, but no difference was found between controls and AD patients.[46] In another study, NRXN-3α, -2α and also Nlgn2, showed what has been described as a biphasic profile, with decreased levels in the preclinical stage and elevated levels in the MCI and dementia stages which could separate these groups from controls.[44] The high dynamics of these proteins at the synapse, and the variability in the time and disease stage of the CSF sampling could account for these contradictory results. Also, in some of the described studies, both sample size and effect size were small.[40,42,43,45] Synapses are probably not the only source of NRXN and Nlgn peptides in the CSF, as more and more studies show that also astrocytes express these proteins.[62-64] The astrocytic expression of NRXNs and Nlgns might be considered as an additional source of variation for the levels of these proteins in the CSF.

NRXN and Nlgn proteins did not correlate with the cognitive decline scored with the MMSE test, which tend to indicate that these proteins do not reflect the synaptic alteration seen in AD. NRXNs and Nlgns moderately correlated with *p*-tau, while there was no correlation with A$\beta_{42/40}$ ratio. The high correlations found with Aβ_{40} and Aβ_{42} are probably reflecting the common proteolytic processing affecting both APP and NRXNs and Nlgns at the synapse.[26,38] With the other synaptic proteins GAP43 and neurogranin, NRXNs and Nlgns showed a moderate to weak correlation, respectively. This might indicate that these proteins reflect different events at the synapse, with both neurogranin and GAP43 being located intracellularly, whereas NRXNs and Nlgns are transmembrane proteins with large extracellular domains communicating with the extracellular environment.

NRXNs and Nlgns have been connected to AD pathogenesis both genetically and at the protein level. The *NRXN-3* gene has been suggested to have a role in sporadic AD susceptibility, with a protective effect in males,[65] while altered expression of the *NRXN-3* was shown in post-mortem frontal gyrus AD brain and has been associated with AD-related changes.[34,66] A frameshift mutation for Nlgn1 has been described in a familial case of AD, which abolished the ability of the protein to exert its function in promoting glutamatergic synapse formation.[67] At the protein level, NRXNs and Nlgns are proteolytically processed by metalloproteases, which cleave their respective extracellular domain leaving the C-terminal fragment as a substrate for γ-secretase, which catalytic units are mutated in familial cases of AD.[68] Therefore, we can hypothesize that other substrates of the protease complex, including NRXNs and Nlgns, are affected by its altered activity.[69] Amyloid-β aggregation in plaques is one of the major hallmarks of AD and according to the amyloid cascade hypothesis accumulation of Aβ peptides is the initial event leading to AD-related changes.[70] Among the different Aβ forms, Aβ oligomers (Aβo) have been described as the most synaptotoxic.[71] Both NRXNs[72] and Nlgns[33,73] have been shown to interact with Aβo at the synapse, an interaction that disrupts their function and is described as one of the possible mechanisms of Aβo toxicity at the synapse. Taken together, these studies make NRXNs and Nlgns interesting candidate biomarkers for synaptic pathology in AD. Moreover, the selective differential distribution of Nlgns in excitatory (Nlgn1) and inhibitory (Nlgn2) synapses[74,75] would allow the simultaneous monitoring of related events in those different synapses. However, results from this study indicate otherwise, suggesting that these proteins do not show a CSF biomarker potential for synaptic impairment related to AD. Yet, this does not preclude their potential as biomarkers for synapse dysfunction and loss in other neurological diseases. Indeed, genetic alteration of NRXNs[76] and Nlgn2,[77,78] -3 and -4[28,79] has been identified in schizophrenia, autism and bipolar disorders and described as a common biological pathway for the synaptic dysfunction aetiology seen in these disorders.[80,81]

The strength of this study lies in the large sample size of the cohort analysed and in the high specificity offered by the PRM method. In addition, straightforward sample preparation and the possibility of multiplexing without the need for antibodies represent an advantage. Further studies can be directed toward the investigation of endogenous peptides (*i.e.*, not digested *in vitro*), which might convey more valuable biomarker information. A more comprehensive characterization of NRXN and Nlgn species present in CSF would probably help in understanding how these proteins are processed outside the CNS and possibly yield a wider range of peptides to investigate as possible biomarkers. However, a major obstacle to the investigation of NRXN and Nlgn proteins is the limited choice of antibodies available and the large size of the proteins themselves, which are excessively long, and thus difficult to analyse by MS without prior proteolytic digestion.

In conclusion, we developed a new robust and specific method for the simultaneous quantification of NRXN and Nlgn extracellular domain peptides in CSF. The possibility to measure multiple synaptic peptides in a panel assay provides a powerful tool to simplify the analysis of the complex biology of NRXNs and Nlgns in AD and other neurodegenerative diseases by detecting changes of those peptides in one assay, using one and the same sample. Further, longitudinal studies would be required to better describe if and how these proteins change in the CSF of patients with neurodegenerative diseases. Finally, this study provides the methodological groundwork to proceed on similar studies in mental disorders in which NRXNs and Nlgns have been shown to be involved (such as schizophrenia and autism), and possibly yield biomarkers for synapse pathology in those diseases.

Contributors

EC, JN, AV and GB designed the study. EC developed the method, performed the experiments, analysed the data and wrote the manuscript. JN and GB supervised during method development and the MS analysis. GB, KB and HZ were responsible for supervision, conceptualization, and verification of the underlying data. AV and CP provided the CSF samples of the clinical cohort. EC, CH participated in diagnosis of the patients and CSF samples collection. GB, BB, AB, HZ, KB contributed to the interpretation of the results and provided critical feedback of the manuscript. All authors have reviewed the manuscript.

Declaration of Competing Interest

HZ has served at scientific advisory boards for Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, Prothena, Roche Diagnostics, and Siemens Healthineers. KB is a cofounder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. CP is a member of the International Advisory Boards of Lilly; is a consultant for Fujiribio, Alzhois, Neuroimmune, Ads Neuroscience, Roche, AgenT, and Gilead; and is involved as an investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck, and Neuroimmune. The other authors declare no competing interests.

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Data sharing statement

Derived data supporting the findings of this study are available in the public, open access, Mendeley Data repository (https://data.mendeley.com/datasets/7sjrs6g6f9/1).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103793.

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