The lipid phosphatase Synaptojanin 1 undergoes a significant alteration in expression and solubility and is associated with brain lesions in Alzheimer’s disease

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Abstract

Synaptojanin 1 (SYNJ1) is a brain-enriched lipid phosphatase critically involved in autophagosomal/endosomal trafficking, synaptic vesicle recycling and metabolism of phosphoinositides. Previous studies suggest that SYNJ1 polymorphisms have significant impact on the age of onset of Alzheimer’s disease (AD) and that SYNJ1 is involved in amyloid-induced toxicity. Yet SYNJ1 protein level and cellular localization in post-mortem human AD brain tissues have remained elusive. This study aimed to examine whether SYNJ1 localization and expression are altered in post-mortem AD brains. We found that SYNJ1 is accumulated in Hirano bodies, plaque-associated dystrophic neurites and some neurofibrillary tangles (NFTs). SYNJ1 immunoreactivity was higher in neurons and in the senile plaques in AD patients carrying one or two ApolipoproteinE (APOE) ε4 allele(s). In two large cohorts of APOE-genotyped controls and AD patients, SYNJ1 transcripts were significantly increased in AD temporal isocortex compared to control. There was a significant increase in SYNJ1 transcript in APOEε4 carriers compared to non-carriers in AD cohort. SYNJ1 was systematically co-enriched with PHF-tau in the sarkosyl-insoluble fraction of AD brain. In the RIPA-insoluble fraction containing protein aggregates, SYNJ1 proteins were significantly increased and observed as a smear containing full-length and cleaved fragments in AD brains. In vitro cleavage assay showed that SYNJ1 is a substrate of calpain, which is highly activated in AD brains. Our study provides evidence of alterations in SYNJ1 mRNA level and SYNJ1 protein degradation, solubility and localization in AD brains.

Keywords: Alzheimer’s disease, SYNJ1, Amyloid β, Tau, Neurofibrillary tangles, Hirano bodies, Phosphatidylinositol
Introduction
Alzheimer disease (AD) is neuropathologically characterized by extracellular amyloid plaques composed of amyloid β (Aβ) peptides and intracellular neurofibrillary tangles (NFTs) constituted of microtubule associated protein tau. AD lesions are tightly related to severe misregulation of cytoskeletal proteins such as microtubule or actin. Hirano bodies, eosinophilic crystal-like structures, are frequently observed in pyramidal neurons of the CA1 area of the Ammon’s horn of AD brains [27]. Hirano bodies are composed of intracellular aggregates of actin, actin-binding proteins, and tau [24]. Smaller inclusions positive for actin-depolymerizing factor ADF/ coflin often occur in linear arrays in AD brains and transgenic AD models [41].

Several studies have shown that lipid phosphatase Synaptotaginin1 (SYNJ1) is profoundly involved in human neurodegenerative diseases such as AD, early onset Parkinson’s disease (PD) and Down syndrome (DS). SYNJ1, originally described as an inositol-5 phosphatase enriched in axon terminals, dephosphorylates phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) at position 5 [39, 60]. SYNJ1 is an essential protein involved in autophagosome and endosomal trafficking [25, 57] and participates in synaptic vesicle recycling [20]. Single nucleotide polymorphisms of SYNJ1 have significant impact on the age of onset of AD [42]. Human SYNJ1 mutations have been reported in familial PD: R268G substitution of SYNJ1 SAC1 domain was identified in early onset familial PD [29, 48, 50]. Homozygous R268G substitution causes Parkinsonian phenotype in knock-in mice [16] and causes presynaptic autophagy defects in flies [57]. SYNJ1 maps to chromosome 21 and SYNJ1 expression is increased in the cortex and in lymphoblastoid cell lines and fibroblasts of individuals with DS [1, 7, 18, 19]. SYNJ1 expression is exacerbated in old individuals with Down syndrome with AD-like neuropathological lesions (DSAD) [38]. Whereas excessive Synj1 expression leads to memory deficits in rodent [59], homozygous Synj1 knockout mice are lethal [20] and a rare human homozygous nonsense mutation in SYNJ1 caused epilepsy and severe tau pathology in a young child [22].

Despite significant implication of SYNJ1 in AD, its localization and expression levels remain unclear in AD brains. There are several controversies as to whether SYNJ1 expression is increased or decreased in AD brains. One study has shown that SYNJ1 protein level is decreased in AD [38] while other studies have reported a significant increase of SYNJ1 in AD brains [42], in association with the APOEε4 allele [61]. In this study, we aimed to analyse the localization and expression level of SYNJ1 protein in human post-mortem brain tissues of non-demented control and AD cases. We found that SYNJ1 immunoreactivity was associated with dystrophic neurites surrounding amyloid plaques where SYNJ1 and the presynaptic marker Synaptophysin were partially colocalized. SYNJ1 immunoreactivity was also detected in actin positive Hirano bodies and in a proportion of the NFTs. SYNJ1 transcripts were upregulated in AD brains, with higher levels in AD patients bearing APOEε4 allele(s) compared to those bearing no APOEε4 allele. SYNJ1 protein was predominantly detected in highly insoluble fractions of AD brains. This study demonstrates that SYNJ1 is significantly mislocalized and misregulated in AD brains.

Materials and methods
Antibodies
Five anti-Synaptotaginin1 antibodies were used in this study (Supplementary Table 1, online resource). Rabbit polyclonal anti-SYNJ1 (HPA011916) was purchased from Sigma. Mouse monoclonal anti-SYNJ1 (BD612249, sc-32,770, TA309245) antibodies were purchased from BD transduction, Santa Cruz Biotechnology and OriGene, respectively. Rabbit polyclonal anti-SYNJ1 ab19904 antibody was purchased from Abcam. Mouse monoclonal anti-Flag M2 (F3165), and mouse monoclonal anti-actin antibodies (A5441) were kindly provided by Dr. Peter Davies (Albert Einstein College of Medicine, NY). Mouse monoclonal anti-Synaptophysin (SY38) was purchased from abcam.

Human brain tissues
Samples from the temporal superior T1 isocortex and hippocampus were obtained from AD and age-matched non-demented control subjects. AD cases were diagnosed according to the National Institute of Aging and Reagan Institute Criteria [9] and scored by neuropathological staging for tau and amyloid pathologies [12, 56]. AD cases including two FAD cases with Amyloid Precursor Protein (APP) or Presenilin1 (PSEN1) mutations and one DSAD were all scored as Braak’s stage V or VI (Supplementary Table 2, online resource). Control cases were non-demented individuals who died without known neurological disorders. The mean ages and post-mortem delays of control cases and of AD patients were not significantly different. Average age at death was 76.8 $\pm$ 1.5 and 75.4 $\pm$ 1.5 years for control ($n = 43$) and AD ($n = 51$) cases respectively (mean $\pm$ SEM) ($p = 0.54$). Average post-mortem delays were 21.8 $\pm$ 2.8 h and 20.1 $\pm$ 1.8 h for control and AD cases (mean $\pm$ SEM) ($p = 0.59$). APOE genotype was determined for the cases with an informed consent for genetic study using PCR amplification for genomic DNA and sequencing as described [55].
Non-demented control and AD individuals were enrolled in a brain donation program of the national network of Brain Bank, GIE NeuroCEB, organized by a consortium of Patients Associations. An explicit consent had been signed by the patient or by the next of kin, in the name of the patient. The project was approved by the scientific committee of the Brain Bank. The whole procedure of the Brain Bank has been reviewed and accepted by the Ethical Committee “Comité de Protection des Personnes Paris Ile de France VI” and has been declared to the Ministry of Research and Higher Education as requested by the French law. Some cases were obtained from ULB LHNN brain bank (BB190052) and were studied in compliance and following approval of the Ethical Committee of the Medical School of the Free University of Brussels.

Animals
The 5XFAD double transgenic mice co-express the human amyloid precursor protein (APP695) carrying the Swedish, Florida, and London mutations and the human PSEN1 carrying the M146L and L286V mutations under thy-1 promoter (Tg6799 line) [47]. The 5XFAD mice were maintained on C57Bl/6J genetic background and only heterozygous transgenic mice were used for this study. Paraaffin embedded brain sections of 12 month-old 5XFAD mice (n = 5) were analysed in this study [33]. Wild-type Wistar rat embryos at embryonic day 17 (E17) were prepared as previously described [34]. All studies on animals were performed in compliance and following the approval of the ethical committee for the care and use of laboratory animals of the Medical School of the Free University of Brussels.

Immunohistochemistry
After formaldehyde fixation (10% buffered formalin), brain tissues were paraaffin embedded and sliced in 7 μm thick sections. DAB staining was performed as previously described [2]. Double immunofluorescence labeling was performed using Tyramide-FITC kit (NEL701A, Perkin Elmer), using a goat anti-rabbit antibody conjugated with biotin (Vector Laboratories, BA-1000) for SYNJ1 detection. Mouse monoclonal antibodies were detected using a goat anti-mouse antibody conjugated to Alexa 568 (A-11031, Invitrogen). Slides were mounted with Fluoromount-G (Southern Biotech) and immunofluorescence labelling was observed with an upright confocal microscope (Olympus Fluoview Fv1000) or with an Axiovert 200 M microscope (Zeiss) equipped with an ApoTome system (Zeiss). For quantitative analysis, SYNJ1 positive neurons and dystrophic neurites in hippocampal CA1–2 pyramidal layer were analysed at 40X images by thresholding analyses using NIH ImageJ as previously reported [58].

**Cell cultures and immunocytochemistry**
HEK 293 cells were grown in DMEM medium F12 supplemented with 10% foetal bovine serum, 100 IU penicillin and 100 μg of streptomycin. For immunocytochemistry, HEK 293 cells grown on PLL-coated cover glasses were transfected with Flag-SYNJ1 1–145 human neuronal isoform (145 kDa) DNA plasmid, a generous gift from Prof. Pietro De Camilli, using Lipofectamine 2000 (Life technologies). Twenty-four hours after transfection, cells were rinsed and fixed (4% paraformaldehyde and 4% sucrose in PBS) for 20 min at room temperature followed by rinses and quenching with 10 mM NH₄Cl in PBS for 10 min. The cells were permeabilized with 0.3% BSA and 0.05% Saponin in PBS at 37 °C for 45 min. After overnight incubation with primary antibodies, the cells were rinsed and incubated with goat anti-rabbit IgG conjugated with Alexa 488 and goat anti-mouse IgG conjugated with Alexa 568 (A-11034 and A-11031, Life technologies).

**RNA extraction and quantitative PCR (qPCR) for SYNJ1 mRNA**
Total RNAs from human T1 isocortex were extracted using Nucleospin RNA II kit (Macherey Nagel, Duren, Germany). The quality and quantity of each RNA preparation were assessed on an Agilent 2100 Bioanalyzer with RNA 6000 NanoChips (Agilent Technologies, Santa Clara, CA, USA). Briefly, RNAs (500 ng) were individually reverse-transcribed into cDNAs for 10 min at 25 °C, then 2 h at 42 °C followed by 5 min at 85 °C using the SensiFAST cDNA synthesis kit (Bioline-Meridian Bioscience, London, UK) according to the manufacturer’s instructions. qPCR gene expression assays were performed in a LC96 system (Roche), in the presence of 1X Lightcycler® 480 Probes Master mix (Roche, France), 200 nM of each primer and 100 nM of specific hydrolysis probe (designed with Universal Probe Library, Roche Applied Science): SYNJ1 5′ ggtttgctcgtaaatctcttg 3′ (forward), 5′tcttgaatttttccaaagacatc 3′ (reverse), PolR2A 5′ caagttcaacagcattg 3′ (forward), 5′ gttgagcttcaagg 3′ (reverse) pPib 5′ ttctctaaccagcagacctgg 3′ (forward), 5′ acctcctgacacatccatc 3′ (reverse) and RNF4 5′ctacagttgacagtctgtgc 3′ (forward) and 5′gatgacacgctcattctgc 3′ (reverse).

SYNJ1 expression was normalized to Peptidylprolyl Isomerase B (pPib), Ring Finger Protein 4 (RNF4) and DNA-directed RNA polymerase II subunit RPB1 (PolR2A).

**Preparation of brain homogenates for biochemical analysis**
About 200 mg of frozen T1 isocortex was homogenised as reported [2, 4] in 10 volumes of ice-cold RIPA buffer containing 50 mM Tris pH 7.4 containing 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 mM EDTA, 1
mM EGTA, Roche complete protease inhibitors, 1 mM PMSE, and phosphatase inhibitor cocktail 2, (Sigma, P-5726) and incubated for 60 min at 4 °C on a rotator. One hundred microliter of the total homogenate was supplemented with Laemmli buffer, sonicated on ice and analysed as the total fraction. The rest of the total homogenates was centrifuged (20,000 x g for 20 min at 4 °C) and the supernatant was used as a RIPA soluble fraction. The RIPA-insoluble pellet was re-suspended by sonication in 5-fold volume of 8 M urea containing protease and phosphatase inhibitors and incubated for 30 min at room temperature on a rotator. The mixture was centrifuged at 20,000 x g at 4 °C for 30 min. The supernatant was used as RIPA insoluble fraction. For each fraction, protein concentrations were estimated by the Bradford method (Bio-Rad) before adding Laemmli buffer.

Sarkosyl fractionation was carried out as previously described [4, 14, 26]. To 1 ml of RIPA soluble fraction of brain lysates at a protein concentration of 2 mg/ml, 10 mg of N-lauroylsarcosine sodium salt (Sigma, L-5125) was added to reach a final concentration of 1% (w/v). The lysates were then incubated at room temperature for 30 min with a mild agitation followed by an ultracentrifugation at 180,000 x g for 30 min at 4 °C. The sarkosyl-soluble supernatant was removed, and sarkosyl-insoluble pellets were rinsed briefly with 500 μl of 50 mM Tris-HCl (pH 7.4) and re-suspended in 200 μl of 50 mM Tris-HCl (pH 7.4) by vigorous pipetting. Sarkosyl-insoluble fractions were analysed by western blotting (WB) and transmission electron microscopy as previously described [3, 4].

Calpain cleavage assays

In vitro cleavage of SYNJ1 by calpain was analysed as previously described [2, 51] in lysates of HEK 293 cells transfected with Flag-SYNJ1 145 kDa neuronal isoform with Lipofectamine 2000. Twenty-four hours after transfection, the cells were harvested in HEPES buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM KCl, 0.1 mM dithiothreitol, complete protease inhibitor tablet). Calpain cleavage was tested in the following conditions: Condition 1- incubation without adding CaCl2; Condition 2 – calpain was activated by adding 2 mM CaCl2 to the lysate; Condition 3 - calpain activation in the presence of 2 mM CaCl2 was inhibited by calcium chelators (2 mM EDTA, 2 mM EGTA); Condition 4 – calpain activation in the presence of 2 mM CaCl2 was blocked by pharmacological inhibitor (400 μM calpain inhibitor I A6185 from Sigma). Cell lysates were kept on ice for 30 min and were vortexed every 10 min for solubilisation. The lysates were then centrifuged at 16,000 x g for 15 min at 4 °C. The supernatant was incubated at 37 °C for 1 h in the 4 conditions detailed above. The reaction was stopped by adding Laemmli buffer followed by incubation at 100 °C for 10 min. The samples were analysed by WB.

Statistical analysis

Statistical significance of comparisons was determined by unpaired Student’s t-tests, by t-test with Welch’s correction for unequal variances or by one-way ANOVA with post-hoc Tukey test using Prism 4 software (Graphpad).

Results

SYNJ1 is accumulated in neurons, plaque-associated dystrophic neurites and Hirano bodies in AD brains

Five anti-SYNJ1 antibodies were characterized. Anti-SYNJ1 HPA011916 antibody provided specific signal in immunostaining and WB and thus was used throughout this study (Supplementary Fig. 1, online resource). Immunohistochemistry was performed using anti-SYNJ1 HPA011916 antibody on paraffin embedded hippocampal sections of human post-mortem brains from non-demented control, AD patients carrying no or APOEε4 allele(s) and DSAD (Fig. 1). In control brains, SYNJ1 immunoreactivity was detected in the neuropil, corresponding to nerve terminals and in the cytoplasm of neurons as previously reported [6, 7, 39, 40, 60] (Fig. 1a). In pyramidal hippocampal neurons in AD, SYNJ1 staining was observed as puncta in neuronal perikarya and in dendrites, in a perinuclear rim and in neuronal processes (Fig. 1b). In pyramidal neurons of the CA1–2, the intensity of SYNJ1 labelling was increased in AD brains compared to control brains (Fig. 1a-c, e). Hirano bodies are often found in CA1 pyramidal neurons in AD brains and

WB

Tissue samples (20 μg/lane) were run in 7.5% Tris-Glycine gels and transferred onto nitrocellulose membranes (sc-3724, Santa Cruz Biotechnology). The nitrocellulose membranes were blocked in 10% (w/v) semi fat dry milk in TBS (Tris 0.01 M, NaCl 0.15 M, pH 7.4) for 1 h at room temperature and were incubated with primary antibodies overnight followed by rinses and an incubation with anti-rabbit (#7074, Cell Signalling Technology, Bioké) or anti-mouse (A6782, Sigma) immunoglobulin conjugated to horseradish peroxidase. After several rinses, the membranes were incubated with SuperSignal West Pico Substrate (Pierce) and were exposed to an X-ray film (Pierce) or to a DARQ-7 CCD cooled camera (Vilber-Lourmat) in a SOLO 4S WL system. Levels of optical density (OD) of protein signals were estimated by densitometry analysis using the NIH ImageJ program. Anti-β-actin immunoblots were used to normalize protein loading.
Fig. 1 (See legend on next page.)
Sphorylated tau in the NFTs (Fig. 2d-l). In CA1 to CA2 surrounded or sometimes co-localized with hyperphosphorylated or donut-shaped forms were also detected and were brains (Fig. 2a-c). SYNJ1 positive structures with granular was detected in non-tangle bearing neurons in AD neurites [8]. A somatodendritic SYNJ1 immunoreactivity was carried out using anti-SYNJ1 and PHF1 antibodies was detected in the axons of plaque-associated dystrophic neurites in AD brain carrying APOE4 allele(s), the intensity of SYNJ1 staining is stronger than in AD cases without APOE4 allele (e). SYNJ1 positive Hirano bodies were also detected (e, arrowhead). A strong SYNJ1 immunoreactivity was observed in plaque-associated dystrophic neurites in CA1–2 area of AD cases carrying APOE4 alleles (f). g Quantification of neuronal SYNJ1 immunoreactivity by image analysis in three groups: control without APOE4 allele, AD group without APOE4 allele and AD group carrying APOE4 allele(s) (n = 3 for each group). Paraffin section of control cases bearing APOE4 allele was not available and was not included in the analyses. SYNJ1 immunoreactivity is significantly increased in AD cases carrying APOE4 alleles compared to AD cases without APOE4 allele. *p < 0.05 and **p < 0.01 by one way ANOVA with post-hoc Tukey test. h Quantification of SYNJ1 immunoreactivity by image analysis in plaque-associated dystrophic neurites. SYNJ1 immunoreactivity is significantly increased in AD cases carrying APOE4 alleles compared to AD cases without APOE4 allele (n = 3 for each group). **p < 0.01 by unpaired t-test. i. In the hippocampus of DSAD brain, a strong immunostaining for SYNJ1 was detected in neuronal perikarya and in dystrophic neurites surrounding amyloid plaques (asterisk). Scale bars 10 μm.

SYNJ1 is accumulated in Hirano bodies
There are two common actin-positive lesions in AD brains: Hirano bodies and much smaller ADF/cofilin rods [41]. To confirm the presence of SYNJ1 immunoreactivity in Hirano bodies, a double-immunofluorescence staining was carried out for SYNJ1 and actin. Actin positive Hirano bodies were systematically immunostained by anti-SYNJ1 antibody but with incomplete overlap: whereas the periphery of Hirano body was strongly stained by anti-actin antibody [36], the centre of Hirano body was strongly labelled by anti-SYNJ1 antibody (Fig. 3a-c). While much smaller actin-positive punctiform staining was remarkable in AD brains, such actin-positive punctiform structures were not immunostained with anti-SYNJ1 antibody (Fig. 3c arrows).

SYNJ1 is partially colocalized with Synaptophysin in plaque-associated dystrophic neurites in AD and 5XFAD brains
SYNJ1 immunoreactivity was detected as globular structures around amyloid plaques in AD brains (Fig. 1d, f). The great majority of SYNJ1 positive structures observed around the amyloid deposits were not directly colocalized with hyperphosphorylated tau (Fig. 3f). Synaptophysin is a presynaptic marker and is accumulated in the axons of plaque-associated dystrophic neurites in AD brains [13] and in 5XFAD mouse brains [52]. SYNJ1 was partially colocalized with Synaptophysin in the plaque-associated dystrophic neurites in AD brains (Fig. 3g-i) and in the 5XFAD mouse brain (Fig. 3j-l). Taken together, these data suggest that SYNJ1 is clearly associated with Alzheimer lesions such as NFTs, Hirano bodies and Synaptophysin-positive dystrophic neurites surrounding amyloid plaques.
**SYNJ1 mRNA is increased in AD brains in association with APOEε4 genotype**

In order to know whether the expression of SYNJ1 is altered in AD brains, **SYNJ1 mRNA level in T1 isocortex was analysed by qPCR in control and AD cases including 2 FAD cases with APP or PSEN1 mutation** (Supplementary Table 2, online resource). There was a significant increase of **SYNJ1 transcripts in AD brains compared to controls** (Fig. 4a). **SYNJ1 transcripts of two FAD cases were in the 95% range and were not distinguishable from sporadic AD cases.** To analyse a possible association of the APOEε4 allele and **SYNJ1 mRNA level, SYNJ1 transcript levels were compared between APOEε4 non-carriers and APOEε4 carriers (one or two alleles) of the control and AD cohorts** (Fig. 4b-c). There was a significant increase of **SYNJ1 mRNA levels in the APOEε4 carriers compared to non- APOEε4 carriers in AD group** (Fig. 4c). Such APOEε4-dependent increase of **SYNJ1 mRNA level was not observed in the control group in our study cohort** (Fig. 4b). There was a significant

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**Fig. 2** Detection of SYNJ1 immunoreactivities in intraneuronal granular structures in AD brains. a-l Double immunostaining for SYNJ1 (a, d, g, j, green) and PHF1 (b, e, h, k, red) shows that SYNJ1 immunoreactivity is detected as a perinuclear rim and in the perikarya in non-tangle bearing neurons (a-c) and in tangle bearing neurons (d-l) in the CA1–2 pyramidal neurons of AD hippocampus. SYNJ1-positive intraneuronal granular or donuts-like structures (g, inset) were occasionally detected in tangle bearing neurons and were surrounded and sometimes overlapped with hyperphosphorylated tau in NFTs (l, arrowhead). Representative images of an APOEε3/3 AD case are shown. Scale bars 10 μm.
correlation between the level of \textit{SYNJ1} mRNA and hyperphosphorylated tau detected with PHF1 antibody by WB (Fig. 4d). These data indicate that there was a significant increase of \textit{SYNJ1} transcripts in AD brains compared to control and that this increase was associated with \textit{APOE}ε4 genotype and tau load in AD cohorts.

SYNJ1 protein becomes highly insoluble and is detected in sarkosyl-insoluble fraction in AD brains
We subsequently analysed SYNJ1 protein expression by WB in brain lysates of T1 isocortex (Fig. 5). First, brain homogenates were sonicated on ice in the presence of Laemmli sample buffer and analysed by WB. In spite of the increased level of \textit{SYNJ1} mRNA, normalized SYNJ1

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**Fig. 3** Detection of SYNJ1 immunoreactivities in Hirano bodies and in plaque-associated dystrophic neurites in AD brains. a-c A double immunofluorescence labelling for SYNJ1 (a, green) and actin (b, red) confirmed that Hirano bodies were immunostained for SYNJ1 in pyramidal neurons of AD brains (c, merge). While SYNJ1 labelling was stronger in the centre of the Hirano bodies (a), actin labelling was stronger in the periphery of Hirano bodies (b). There were numerous smaller punctiform structures that were actin positive but SYNJ1 negative in AD brains (arrows). d-f A double immunofluorescence labelling for SYNJ1 (d, green) and PHF1 (e, red) does not show association of SYNJ1-positive globular structures and hyperphosphorylated tau in the dystrophic neurites surrounding senile plaques. g-l A double immunofluorescence labelling for SYNJ1 (g, j, green) and Synaptophysin (SY38, h, k, red) shows partial colocalization of SYNJ1 and Synaptophysin (arrowheads) in AD brain (g-l) and in 5xFAD mouse brains at 12 months (j-l). Some plaque-associated dystrophic neurites were yet devoid of SYNJ1 immunoreactivity (l, asterisk). Representative images of an \textit{APOE}ε3/3 AD case (a-l) and 5xFAD (j-l) are shown. Scale bar 10 μm for a-c and 40 μm for d-l.
protein level (145 and 170 kDa isoforms) was significantly decreased in total fraction of AD brains (Fig. 5b). We wondered whether SYNJ1 might become highly insoluble in AD brains and might not be completely detectable in our homogenisation protocol. Fractionation and urea solubilisation were carried out in order to verify potential changes in SYNJ1 partitioning between soluble and insoluble fractions and to better solubilize insoluble proteins (Fig. 5c-d). The level of SYNJ1 in the RIPA-soluble fraction was decreased in AD brains (Fig. 5c). SYNJ1 protein was increased in the AD cases carrying one or two \( APOE^\epsilon 4 \) allele(s) compared to AD cases carrying no \( APOE^\epsilon 4 \) allele (Fig. 5c'). On the contrary to the RIPA-soluble fraction, the level of SYNJ1 protein detected in the RIPA-insoluble fraction containing largest protein aggregates was significantly increased in AD brains (Fig. 5d). There were some cleaved bands of SYNJ1 clearly detected in AD brains in the RIPA-insoluble fraction approximately at 50, 80 and 100 kDa (Fig. 5d). Cleaved fragments of SYNJ1 were less frequently observed in the RIPA-insoluble fraction of control brains. Some AD cases exhibiting severe tau load contained SYNJ1 positive smears at higher molecular weight than 145 and 170 kDa in RIPA-insoluble fraction (Fig. 5d). There was a significant correlation between the levels of SYNJ1 mRNA and phosphorylated tau detected by WB using PHF1 (\( r^2 = 0.3713, n = 66, p = 0.0021 \), by Pearson correlation test).

**SYNJ1 is a substrate of calpain**

There were several bands below 145 kDa that were detected by SYNJ1 antibody in the RIPA-insoluble fraction of AD brains (Fig. 5c). Such SYNJ1-positive bands might
Insoluble SYNJ1 is increased in AD brains and is correlated to tau load. 

**a** Summary of the fractionation protocol used to obtain total, RIPA-soluble, RIPA-insoluble and sarkosyl-insoluble fractions. **b** SYNJ1 was significantly decreased in the total homogenate of AD T1 isocortex. SYNJ1 was significantly decreased in RIPA-soluble fraction of AD brains. There was a significantly higher level of SYNJ1 protein detected in the AD cases carrying APOE4 allele(s) compared to the AD cases without APOE4 allele. **d** SYNJ1 was increased in RIPA-insoluble fraction of AD brains. Lower MW SYNJ1 positive bands were detected approximately around 100 kDa, 80 kDa and 50 kDa in the AD brain. For **b-d**, T1 isocortex from control (n = 42) and AD (n = 50) including 2 FAD cases with APP or PSEN1 mutation were analysed. **p < 0.01, *** p < 0.001, by unequal variances t test.**

There was a significant positive correlation between the levels of SYNJ1 protein and phosphorylated tau detected using PHF1 in the RIPA-insoluble fraction. ($r^2 = 0.1443, n = 92, p = 0.0002$, by Pearson correlation test).
result from proteolytic cleavage by proteases such as calpain abnormally activated in AD brains [31, 53]. To test whether SYNJ1 is proteolysed by calpain, HEK 293 cells were transiently transfected with Flag-SYNJ1 145 and cultured for 24 h. The cell lysate was incubated at 37 °C for 1 h in the presence or absence of calcium. In the presence of calcium in the lysate, SYNJ1 was significantly decreased by 50% and a 140-kDa band appeared (open circle). The proteolysis was inhibited by adding calcium chelators (EDTA and EGTA) or calpain inhibitor I. The graph shows the OD of SYNJ1 normalised to actin in each condition of three independent experiments. *p < 0.05 by one-way ANOVA with post-hoc Tukey test.

**Discussion**

In the present study, we assessed the changes in SYNJ1 localization and expression level in post-mortem AD brains. SYNJ1 is co-enriched with PHF-tau in sarkosyl-insoluble fraction. a WB for SYNJ1 and PHF1 in sarkosyl-insoluble fraction of control (n = 3) and AD (SAD n = 3, FAD with APP mutation n = 1). SYNJ1 was enriched in sarkosyl-insoluble fractions with PHF-tau. b Representative image of the sarkosyl-insoluble PHF-tau of a sporadic AD case taken by transmission electron microscopy. Scale bar 0.2 μm.

Fig. 6 SYNJ1 is co-enriched with PHF-tau in sarkosyl-insoluble fraction. a WB for SYNJ1 and PHF1 in sarkosyl-insoluble fraction of control (n = 3) and AD (SAD n = 3, FAD with APP mutation n = 1). SYNJ1 was enriched in sarkosyl-insoluble fractions with PHF-tau. b Representative image of the sarkosyl-insoluble PHF-tau of a sporadic AD case taken by transmission electron microscopy. Scale bar 0.2 μm.

Fig. 7 In vitro cleavage assay for SYNJ1 by calpain. a HEK 293 cells were transiently transfected with Flag-SYNJ1 145 and cultured for 24 h. The cell lysate was incubated at 37 °C for 1 h in the presence or absence of calcium. In the presence of calcium in the lysate, SYNJ1 was significantly decreased by 50% and a 140-kDa band appeared (open circle). The proteolysis was inhibited by adding calcium chelators (EDTA and EGTA) or calpain inhibitor I. The graph shows the OD of SYNJ1 normalised to actin in each condition of three independent experiments. *p < 0.05 by one-way ANOVA with post-hoc Tukey test.

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SYNJ1 is associated with Hirano bodies, NFTs and dystrophic neurites in AD brains

Hirano bodies, eosinophilic crystal-like structures, are frequently observed in pyramidal neurons of the CA1 area of the Ammon’s horn of AD brains, other neurodegenerative diseases and during aging [27]. Hirano bodies are composed of intracellular aggregates of actin and actin-binding proteins [24, 35]. We observed that SYNJ1 was partially colocalized with actin, which is highly accumulated in Hirano bodies. SYNJ1 is involved in actin organization in the cell via regulation of PIP2 that binds to actin-regulating proteins [54]. The proline-rich domain of SYNJ1 binds to SH3 domains of several actin regulating proteins such as Myosin 1E [30] or DAP160/intersectin [28]. The presence of the latter proteins in Hirano bodies has not been documented to our knowledge and further analyses are necessary to uncover the roles of SYNJ1 and its binding-partners in Hirano body formation. Reduction of soluble SYNJ1 and its proteolysis by calpain may lead to its dysfunction and may be responsible for a disorganization of the actin network leading to the formation of Hirano bodies. Actin positive rod shaped structures can be induced in cultured hippocampal neurons by oxidative stress or Aβ treatment [41], and are also observed in tau transgenic rTg4510 mouse brains [23] and in APP transgenic Tg2576 mouse brains [37]. How these small actin positive rods are related to Hirano body formation remains largely unclear. Our data show that SYNJ1 is associated only to Hirano bodies but not clearly to other actin-positive structures, suggesting that the association of SYNJ1 with Hirano bodies is specific.

SYNJ1 is enriched in the insoluble fraction of AD brains

Despite the increase of SYNJ1 mRNA, SYNJ1 protein level in the total and RIPA-soluble fractions was decreased in AD brains. Our observation on the reduction of SYNJ1 in the total and RIPA soluble fractions is consistent with a previous study reporting a decrease of SYNJ1 protein level in the 1% SDS soluble fraction of AD brain lysates [38]. The reduction of SYNJ1 in soluble fraction might be caused by multiple mechanisms of post-transcriptional and/or post-translational modifications of SYNJ1. Firstly, SYNJ1 may be sequestered by insoluble PHF-tau and may be trapped into NFTs. This hypothesis is supported by our results on co-enrichment of PHF-tau and SYNJ1 in sarkosyl-insoluble fraction and partial co-localization of SYNJ1 and hyperphosphorylated tau in NFTs. The smear-like migration pattern of SYNJ1 on SDS-PAGE that we observed in RIPA-insoluble fraction was quite similar to that of aggregate-prone proteins such as tau or TDP-43 [43]. Secondly, post-translational modifications (e.g. proteolysis, phosphorylation, oxidation etc.) of SYNJ1 may further modify SYNJ1 solubility. We demonstrated that SYNJ1 is a substrate of calpain, a protease highly activated in AD brains [53]. A number of neurotoxic factors, including Aβ, can activate calpain [32]. Calpain may be involved in the reduction of SYNJ1 in the total fraction of AD and may also be involved in the formation of cleaved fragments of SYNJ1 detected in the RIPA-insoluble fractions of AD brains. We cannot exclude the possibility that other proteases may as well be involved in SYNJ1 cleavage. Further analyses on post-translational modification including phosphorylation or oxidation of SYNJ1 are necessary to uncover the roles of SYNJ1 in AD brains.

Cases and higher in AD patients bearing APOE ε4 allele(s) were significantly increased in AD brains and higher in AD patients bearing APOE4 allele(s) compared to those bearing no APOEε4 allele. By biochemical fractionation, we provide evidence that SYNJ1 was co-enriched with PHF-tau in sarkosyl-insoluble fraction in AD brains. SYNJ1 was predominantly detected in the insoluble fraction in AD brains and several cleaved SYNJ1 fragments were also observed in the RIPA-insoluble fraction. Results from in vitro cleavage assays showed that SYNJ1 is a proteolytic substrate for calpain, which is activated in AD brains. These results constantly support our hypothesis that SYNJ1 is significantly misregulated in AD brains.

PIP2 is a substrate of SYNJ1 and plays important roles in many cellular signalling pathways [21]. PIP2 is accumulated and enriched in NFTs and granulovacuolar degeneration [45] and is colocalized with tau kinases such as CDK5 or GSK3β [44]. Immunolabelling of SYNJ1 positive granular or donut-like structures were clearly observed in perinuclear rim and perikarya of NFTs of AD brains. A molecular interaction between SYNJ1 and PHF1-positive phosphotau are supported by our results of partial colocalization in NFTs, co-enrichment in sarkosyl-insoluble fraction and a significant correlation between SYNJ1 and phosphotau in RIPA insoluble fraction.

SYNJ1 was also accumulated in dystrophic neurites around amyloid deposits. Presynaptic terminal swellings can be found in the corona of the senile plaques, where Synaptophysin [13], APP [11] or ADF/cofilin accumulate [41]. Taken together, we report for the first time a close association of AD neuropathological lesions and SYNJ1 in post-mortem AD brains.
necessary to decipher other potential mechanisms underlying solubility change of SYNJ1 observed in AD brains.

SYNJ1 and APOE4 in AD brains
Our study provides the first evidence that SYNJ1 immunolabelling in neurons or in plaque-associated dystrophic neurites was increased in AD cases carrying one or two APOE4 alleles compared to those carrying no APOE4 allele. SYNJ1 transcripts were significantly increased in AD brains compared to age-matched controls. Increase of SYNJ1 mRNA in APOE4 carriers in our AD cohort is consistent with the previously reported upregulation of SYNJ1 mRNA in APOE4 carriers [59, 61], supporting the hypothesis that SYNJ1 is involved in the APOE-related AD susceptibility.

SYNJ1 misregulation and AD
Our data shows that SYNJ1 undergoes significant alterations in expression, solubility and subcellular localization in AD. Increased level of insoluble SYNJ1 in AD brains may induce toxicity and could be associated with synaptic dysfunction and eventually with cognitive deficits as previously reported [59, 61]. Misregulation of SYNJ1 in AD may contribute to excitotoxicity [49], dysregulation of endocytosis [17] and deficits in autophagy [5, 46], all defects reported in AD patients and models.

Reduction of SYNJ1 has been proposed as a therapeutic target for amyloid-induced toxicity [10], amyloid clearance [62] and tau pathology after traumatic brain injury [15]. Nevertheless, a complete or partial loss of function of SYNJ1 protein may lead to profound pathological effects. Synj1-deficient mice die shortly after birth [20] and a homozygous truncating mutation within SYNJ1 resulted in a neurodegenerative tauopathy associated to a severe reduction in SYNJ1 protein level in human [22]. In addition, recent studies on knock-in models of an early-onset PD mutation of SYNJ1 (R258Q) suggested critical roles of SYNJ1 for endocytic protein dynamics and for balancing excitatory and inhibitory transmission [16] as well as autophagosome maturation at presynaptic terminals [57].

Our present study provides strong evidence that SYNJ1 is misregulated in AD brains and points to the possibility that targeting abnormal modifications of SYNJ1 could open up new windows to novel therapeutic strategies against AD.

Conclusions
These data strongly support the hypothesis that SYNJ1 undergoes significant alterations in its localization and solubility in AD brains.

Supplementary information
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Authors’ contributions
KA, MN, ST, GF, GP, LD, ZY, VS, SM, MA and RDD performed analyses on WB, QPCR and IHC. KA, KL, BD, CD, MCP and JPB drafted the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
Data generated during this study are included in this published article and its supplementary information files.

Ethics approval
Autopsies and gene analyses were carried out after written informed consent was obtained from family members. All experiments in this study were approved by the ethical committees of the GIE Brain Bank and the Medical school of ULB.

Consent for publication
Family members have consented to publication.

Competing interests
The authors declare that they have no competing interests.

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