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Expression Patterns of C-Menin and Cholinergic Synaptic Machinery in Human Autopsied Brains from Alzheimer's and Controls

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Abstract

The evolutionarily conserved tumor suppressor gene *MEN1* (multiple endocrine neoplasia type 1) and its encoded protein menin, have recently been shown to regulate cholinergic synapse formation in both vertebrates and invertebrates. Moreover, its knockout in adult animals results in learning/memory deficits and depressive-like behaviors in rodent models in a manner analogous to that of the Alzheimer. However, menin's presence has not been demonstrated in the human brain, nor has its characterization been documented in the neurodegenerative model. In this study, we sought to document the presence of menin, synaptophysin, postsynaptic density protein-95 (PSD-95), nicotinic acetylcholine receptors (nAChRs) and tau in autopsied human tissue obtained from controls followed by the examination of menin's expression patterns in the Alzheimer disease (AD) individuals. We used frozen tissue obtained from Calgary Brain Bank, controls (n = 6) and AD (n = 6). Optimized immunohistochemistry protocols were used to define the patterns of co-expression of menin with AD marker, tau. For the first time, our data document menin immunoreactivity in human brain and demonstrate its localization patterns in the controls and AD human brains. Moreover, we demonstrate that in the human hippocampus, menin expression is likely perturbed in the AD group. Further investigation is required to determine if perturbation of menin function may underlie nicotinic receptor dysfunction mediating learning and memory deficit in the AD brain.

Keywords: *C-Menin; Tau; PSD-95; Synaptophysin; nAChR; MAP2; Alzheimer's Disease*

Introduction

Alzheimer's disease (AD) affects 50% of all dementia patients and in the next decade it is expected to afflict 42 - 50 million people worldwide [1]. The age-related degeneration involves neurons in certain brain regions - primarily in the hippocampus involving cholinergic synaptic function. Multiple factors likely affect AD pathologic changes and a loss of neuronal structures and their communications [1]. However, none of these factors or mechanisms have been shown conclusively, to fully account for AD pathology. A few studies have suggested that a deficiency in the cholinergic signaling may initiate the progression of the disease, whereas others have proposed that either

hyperphosphorylated tau protein or amyloid beta (A-beta) trigger a cascade of events leading to AD pathology [1-3]. To date, however, all clinical trials targeting amyloid or tau aggregation have failed to significantly alter the course of the disease [4] which indicates that the precise causes and mechanisms underlying AD remain to be fully defined.

A loss of cholinergic function and synaptic connections in the hippocampus is considered to be one of the most important neuropathological hallmarks of dementia in AD [5,6] and this cascade, in turn is thought to precede the deposition of β-amyloid plaques and neurofibrillary tangles [1]. Soluble oligomeric β-amyloid alters nicotinic acetylcholine receptors (nAChR) function, resulting in the disruption of nAChR-dependent plasticity at glutamatergic synapses of the hippocampus [7-9]. Furthermore, several other studies have shown that a decrease in the expression of hippocampal brain-derived neurotrophic factor (BDNF) is commonly observed in AD [10,11]. Notwithstanding the extensive evidence in support of cholinergic hypothesis of AD, pharmacological perturbations aimed at augmenting either acetylcholine release or nAChR function have however, met with little success [12]. These studies suggest that pathways upstream of the nAChRs might potentially be responsible for the disruption of cholinergic transmission in AD, though direct evidence is still lacking.

We have recently demonstrated that *MEN1* gene (Multiple Endocrine Neoplasia type 1), which has long been known as a tumor suppressor in humans, also plays an important role in the formation and maintenance of cholinergic synapses in both invertebrates and vertebrates where its expression patterns are developmentally regulated [13-15]. Menin, encoded by *MEN1* gene, has since been shown to regulate the expression of nAChRs in a trophic factor mediated and activity-dependent manner, and that it's *in vitro* perturbation in the hippocampus disrupts the targeting of nAChRs localization and function [13,14]. Moreover, *in vivo* conditional knockout of *MEN1* in a mouse model results in learning, memory and cognitive deficits, as well as depressive like behaviors [16,17].

The involvement of evolutionarily conserved and developmentally regulated tumor suppressor genes, such as *MEN1* [18] and *DLG1* (*SAP97*) [19], in the regulation of cholinergic and glutamatergic synaptic structures and functions is well documented. However, with the exception of its extensive involvement in the endocrine tumor suppression, the menin presence in the human brain has not yet been documented, nor has its association with other elements of the cholinergic synaptic machinery demonstrated.

In this study, we asked the question whether menin is expressed in human hippocampus in conjunction with other elements of the cholinergic synaptic machinery. We also investigated whether menin exhibits any close proximation with hyperphosphorylated tau, which is abnormally aggregated early in AD. Using human AD and non-AD hippocampal sections from the autopsied brain, we provide evidence that menin is localized to hippocampal neurons along with other elements of the synaptic machinery, such as postsynaptic density protein-95 (PSD-95), synaptophysin and nAChRs. We also provide morphological evidence demonstrating juxtaposition of menin with hyperphosphorylated tau protein.

Methods

Subjects and medical history

Post-mortem specimens from AD patients (n = 2) and age-matched N-AD (n = 2) were obtained from the Calgary Brain Bank after pathological characterization. Frozen hippocampi from AD patients (n = 4) and age-matched N-AD (n = 4) were obtained from the Calgary Brain Bank (CBB) for molecular characterization of menin protein and its fragments, C-menin and N-menin. The tissue was screened for Alzheimer's disease, synucleinopathies, tauopathies, frontotemporal dementias, and vascular disease, and stained with beta-amyloid, tau, alpha-synuclein, and TAR DNA-binding protein 43 (TDP-43) for further validation of any given pathology.

Tissue fixation and processing

For tissue sectioning, formalin-fixed, paraffin-embedded (FFPE) sections were cut at five microns thickness with Sakura Accu-edge low profile microtome blades on Leica RM2245 microtomes. Sections were floated on a warm water bath to help remove wrinkles, and

then picked up on glass microscope slides (Leica Apex Superior Adhesive slides 1" x 3" x .04"), blotted, and baked for two hours at 65°C in preparation for staining. For molecular analysis, the hippocampal frozen sections which contained the dentate gyrus, CA3, CA2, and CA1 regions, and the end of the subiculum were used for protein extraction.

The Bond Polymer Refine Detection system (Leica Biosystems) was used to detect antigens using monoclonal and/or polyclonal antibodies. This controlled polymerization technology prepared polymeric horseradish peroxidase (HRP)-linker antibody conjugates. The specimen was incubated with hydrogen peroxide to quench endogenous peroxidase activity. The substrate chromogen, 3, 3'-diaminobenzidine tetrahydrochloride (DAB), was used to visualize the complex via a brown precipitate. Hematoxylin (blue) counterstaining allowed for the visualization of cell nuclei. The enzyme alkaline phosphatase (AP) and its substrate Fast Red chromogen was used to visualize the complex via a pink precipitate and was used for dual staining with DAB.

Immunohistochemistry protocol

The IHC staining was carried out on Dako Omnis auto-stainer (Agilent, Santa Clara, CA, USA) at Alberta Precision Labs (APL) as a routine procedure. Five microns FFPE sections were pretreated with target retrieval buffer with high pH (Agilent) followed by a series of staining and washing steps. The slides were subjected to a series of deparaffinization (xylene I, II, III), removal of xylene (ethanol 100%, I, II, III) and hydration (ethanol 95%, ethanol 70%, distilled water). The mouse monoclonal PSD-95 primary antibody (Antibodies Incorporated, 75-028, 1/500 dilution), rabbit polyclonal C-menin primary antibody (Bethyl Laboratories, A300-105A, 1/200 dilution), rabbit polyclonal MAP2 primary antibody (fischer scientific, AB5622MI, 1/300 dilution), mouse monoclonal Tau- PHF antibody (Abcam, Y103799, 1/500 dilution) and the mouse monoclonal synaptophysin primary antibody (AbCam, ab52636,1/5000 dilution) were diluted using Dako antibody diluent. The slices were incubated for twenty minutes for both primary and secondary antibodies. DAB+ Substrate Chromogen system (Agilent, Santa Clara, CA, USA) was used as post incubation detection reagent. Hematoxylin was applied for counterstaining for nuclear staining. The specimens were then mounted using Flo-TEXX mounting medium with (Lerner Laboratories). For the α7 nAChRs, Alex555-conjugated Bungarotoxin was diluted at 1/50 dilution. Two-hours incubation period was followed by DAPI counter staining. The slides were mounted with APRL mounting medium and sealed with nail polishing material.

Imaging/processing/analysis

The VS120 (100-slide), a high throughput whole slide scanner in brightfield mode, was used at 40X. Cellsens software processing was used for area fraction measures to quantify the expression. Nikon A1R-MP Multiphoton Microscope, a visible laser confocal scanner at 60X, with a visible range of 400-750 nm and 2.5/5/10 nm variable resolution was used to visualize the fluorescent signal of α7 nAChR. QuPath pixel classifier training program was used to measure the expression of single stains for DAB (C-menin), MAG (tau) and hematoxylin (nuclei) followed by pixel overlap quantification between DAB and MAG to determine the extent of co-localization.

Protein extraction and Western blotting

Frozen human hippocampi were provided by the CBB which included the CA1, CA2, CA3 areas and the subiculum. Protein sample preparation (stored at -80°C) and Western blotting (WB) were performed on the samples. Blocking and antibody incubations of WB PVDF membranes were performed with 5% skim milk powder + 0.1% Tween-20 in 1 × PBS for 1h at room temperature or overnight at 4°C and menin C-terminal epitope [Bethyl Laboratories, 1:2000]; menin N-terminal epitope [Santa Cruz Biotechnology, 1:2000]; actin [Sigma-Aldrich, 1:2000]; IRDye-800CW and IRDye-680 conjugated α-mouse or α-rabbit IgG [Li-Cor biosciences, 1:5000] were used as primary and secondary antibodies. WB membranes were visualized with a Li-Cor Odyssey infra-red imager and the bands were analyzed with the gels tool on ImageJ.

Experimental design and analysis

Data sets were derived from ≥ 3 independent experiments for reliability and replicability. QuPath software was used for image processing, pixel overlap and calculating the degree of colocalization. Equal areas for the region of interest (ROI) were selected randomly

(independent of the size of the tissue) to minimize sampling bias. The parameters for the quantification tools were kept consistent for all cases. The mean values were calculated for pixel overlap and western blot results for comparing the N-AD and AD cases.

Results

This study was undertaken to determine whether menin a synaptic protein, encoded by *MEN1* gene is present in human autopsied tissue obtained from both "normal" and AD brains. We wanted to decipher its localization patterns along with other synaptic proteins such as postsynaptic density protein-95 (PSD-95), synaptophysin (presynaptic) and non-synaptic tau protein in both normal and AD brains. *MEN1* gene and its encoded protein menin are evolutionarily conserved across many species and play an important role in learning and memory in animal models [13-15]. Perturbations of *MEN1* have been shown to generate a phenotype which exhibits learning and memory deficit in a mouse model [16]. However, its presence in human brain and co-localization with other synaptic proteins has not yet been shown. Moreover, it is also unknown whether menin expression patterns are altered in an AD brain, and if either directly or indirectly it could be corroborated with AD etiology. Human autopsied FFPE brain sections were obtained from Calgary Brain Bank, sliced into 5-micron sections and the tissue integrity tested using stains for various synaptic proteins.

Neuronal localization in a control autopsied human tissue regions of entorhinal cortex (EC) and hippocampus (Hip)

Microtubule associated protein 2 (MAP2) stabilizes microtubules in the axons and dendrites of post-mitotic neurons [20] and is considered a reliable marker of neuronal processes. To assess the integrity of the human brain tissue, and the neurons in various hippocampal and cortical regions, the sections were labeled with MAP2 antibody conjugated with DAB marker, and the nuclei were stained with hematoxylin (blue). MAP2 antibody primarily not only tagged neuronal processes but also the cell bodies leading into the proximal dendrites. These data demonstrate that the tissue integrity was not compromised during autopsies, tissue sectioning, subsequent fixation procedure, and that the specificity of the antibody staining was also validated. Figure 1 shows the expression patterns of MAP2 protein which primarily stained the axons and dendrites of neurons (Ai, ii and iii).

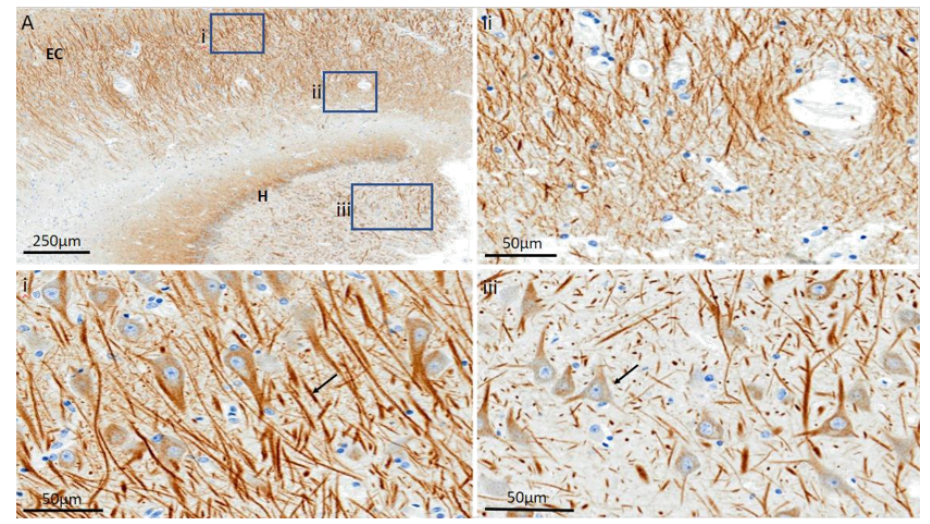

Figure 1: *A) Distribution patterns of MAP2 protein in the hippocampus (H) and the surrounding entorhinal cortex (EC) of control human tissue. i) MAP2 is localized to both neuronal and axonal/dendritic cytoplasm, the proximal dendrite (arrow) and fine neurites in the matrix. ii) MAP2 expression in the neuritic projections in the stratum radiatum. iii) In CA3 region of the hippocampus (the black arrow points to a MAP2 expression in a neuronal cell body).*

Distribution and localization of presynaptic (SNP) and postsynaptic protein (PSD-95) in a control aging human tissue

We next to sort to determine the precise localization and distribution patterns of two major synaptic proteins in both human hippocampus and cortical regions. As described above, the fixed tissues were sectioned and stained with synaptophysin and PSD-95 antibodies that were optimized through a series of optimization steps as described in the methods section. Hematoxylin was used to stain the nuclei of both neurons and the non-neuronal cells. We discovered an interesting staining pattern for synaptophysin, which is an integral membrane protein that makes up the pore complex at the presynaptic membrane [21] throughout various regions of the brain. Specifically, slices were labeled with the synaptophysin antibody conjugated with DAB marker, whereas the cell bodies were labeled blue with nuclear stain hematoxylin. The cell bodies and the initial axon segments of neurons were generally devoid of any staining in both regions of the brain examined (Figure 2).

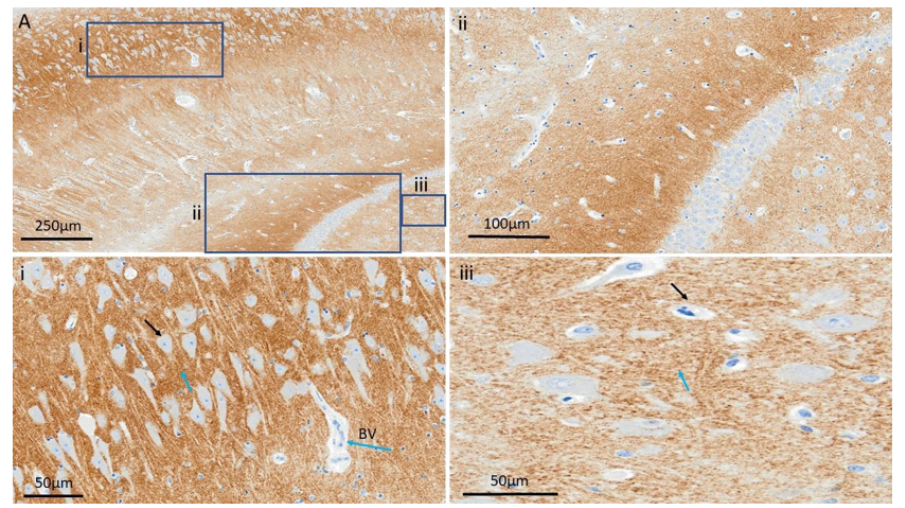

Figure 2: *A) Distribution patterns of synaptophysin protein in the hippocampus of control human tissue. i) The black arrow points to the cell body and the initial axon segments, which are devoid of synaptophysin staining compared to neuritic and dendritic, extrasomal regions (blue arrow). Blood vessel (BV). ii) The neuronal cell bodies were devoid of synaptophysin. iii) The black arrow indicates the neuronal cell body, which is devoid of synaptophysin staining, whereas the blue arrow shows the expression patterns of synaptophysin in the neurite projections within the hippocampus.*

The data demonstrates that synaptophysin is primarily localized to extra-somal regions of neurons whereby the cell bodies were primarily devoid of this protein. This approach further validated our ability to not just visualize larger proteins such as neurofilaments, but also smaller proteins such as synaptophysin which are essential components of the presynaptic machinery. These results thus attest further our ability to maintain tissue integrity at the resolution of synapses and synaptic proteins.

Postsynaptic density protein-95 (PSD-95), a member of the membrane-associated guanylate kinase (MAGUK) family, is a commonly used marker for identifying the postsynaptic neurons [22]. The optimized PSD-95 antibody was used to decipher the precise distribution patterns of PSD-95 in both hippocampus and the cortex. The antibody labeled the somata and the axonal/dendritic processes in both parts of the brain tissue (Figure 3). In contrast to synaptophysin however, we found that the PSD-95 labeled not just the somata but also both the dendrites and the axons through their entire length.

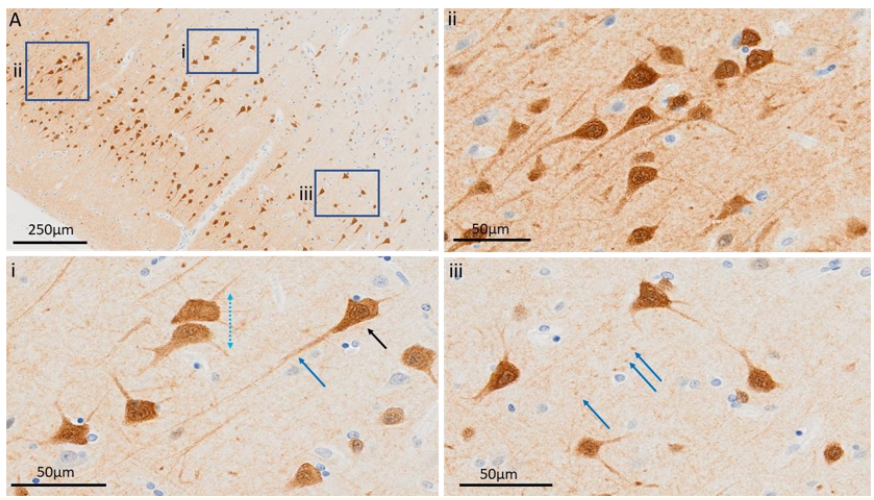

Figure 3: *A) Distribution patterns of PSD-95 protein in the hippocampus of control human tissue. i) The blue dashed line shows PSD-95 localization in dendrites whereas black and blue arrows show somata and axonal distribution of this protein respectively. ii) The figure depicts another area magnified to show a similar pattern of PSD-95 as shown in (i) albeit with more densely packed neurons with greater corresponding antibody staining. iii) The blue arrows indicate putative postsynaptic boutons. The glial cell bodies were also labeled with the nuclear stain DAPI.*

We next attempted for the first time, a double staining approach for both PSD 95 and SNP in the same tissue in order to decipher their localization and distribution patterns (See figure 4). It was interesting to observe a clear labeling of SNP and PSD-95 in distinct layers of the cortex whereas this demarcation became difficult to decipher due to intense axonal and dendritic density with there being extensive overlap in the entorhinal cortex. The PSD-95 labeled processes were clearly discernible whereas the entire region was also intensely stained with SNP. Figure 4 shows clearly that PSD-95 somata and the entire length of their axons whereas synaptophysin labeled cell bodies were devoid of staining. Extensive (pink) staining was however discernible throughout the tissue. Notwithstanding the potential color mixing, we were however, able to decipher co-localization of both proteins throughout the brain slices - especially in the areas that were devoid of cell bodies. Taken together, these data demonstrate our ability to successfully label both pre- and the postsynaptic proteins in the same tissue obtained from autopsied human brain.

C-menin is expressed in both hippocampus and entorhinal cortex (EC)

Menin protein, the product of the *MEN1* tumor suppressor gene, is responsible for the transcription and synaptic clustering of nAChRs in invertebrate and vertebrate central neurons and is shown to play an important role in the regulation of nAChR targeting at synapses in an activity and trophic factor-dependent manner [13,14]. More recently, it was also shown to regulate several aspects of hippocampus-dependent learning and memory in a mouse knockout model [16]. We had previously demonstrated that menin protein gets cleaved in an activity-dependent manner into two fragments, C-menin and N-menin. The calpain-dependent C-terminal fragment (C-menin) was shown to regulate the clustering and targeting of α7 subunit-containing nAChRs at the glutamatergic presynaptic terminals, and to cause nicotine-induced presynaptic facilitation [14]. The N-menin fragment on the other hand, was found responsible for the regulation of transcription of the MEN 1 gene at the nuclear level [14]. However, the presence of either of these fragments in the adult human brain -

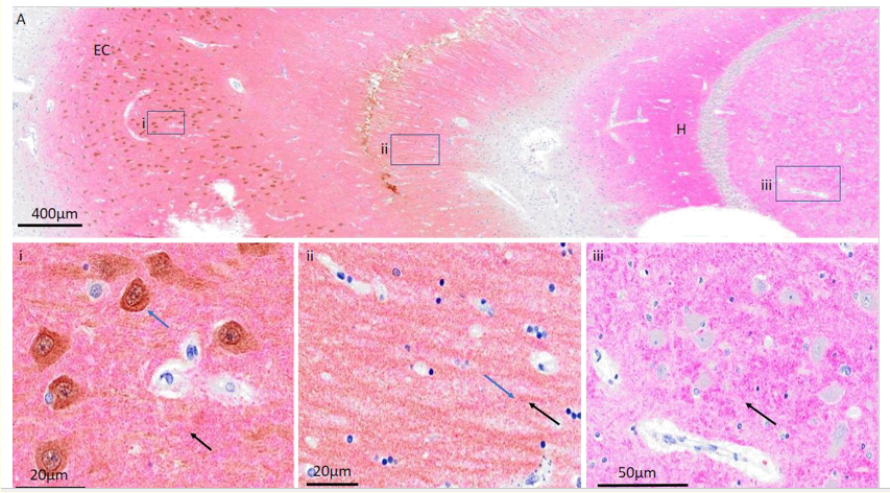

Figure 4: *A) Distribution patterns of PSD-95 and synaptophysin proteins in the hippocampus (H) and surrounding entorhinal cortex (EC) of control human tissue where the expression of PSD-95 is being indicated by the color brown, and synaptophysin is shown as pink. i) The blue arrow points to neurons expressing PSD-95 (dark brown), whereas the black arrows highlight the neuritic/dendritic areas expressing synaptophysin in EC (pink). ii) The blue and the black arrows point to the potential overlap between PSD-95 and synaptophysin in EC. iii) The inner layers of the hippocampus primarily exhibited synaptophysin staining (black arrow) with there being little PSD-95 staining.*

specifically - the C menin - has not yet been demonstrated. To test for its presence in the human brain, the slices were prepared as above and an optimized menin antibody was used to label various brain regions. It is important to note that the specificity of menin antibody has recently been validated throughout mouse development and its expression patterns were deduced in both neurons and the glial cells [15]. Figure 5 shows the localization and distribution patterns of C-menin both in the somata and their processes in a manner analogous to that of observed previously in a rodent model [14,15]. These data provide direct evidence that indeed C-menin protein is expressed in human brain and that this expression is neuron/glia specific.

Menin has previously been shown to be specifically co-localized with nAChRs where it regulates their expression patterns and targeting in the mouse hippocampus [14]. We next sought to define the expression patterns of nAChR subunit α7 in the hippocampus of control human brain. Homomeric α7 nicotinic acetylcholine receptors (nAChRs) are expressed throughout the hippocampus, and nicotinic signaling plays an important role in learning and memory [23]. In the present study, the distribution patterns of α7 nAChRs were observed in the hippocampus, as shown in figure 6. The AChRs appeared to form clusters around the neuronal processes (most likely the dendrites) and were well distributed throughout the hippocampus. These data demonstrate that the α7 nAChRs are indeed present at the synaptic sites where they appear to form cluster like aggregates.

Expression of Tau PHF protein in hippocampus and entorhinal cortex of normal aging and AD brains

We next sought to determine, whether brain slices from control humans also labeled for proteins that are hallmark of AD. Tau-PHF is a commonly used stain for demonstrating the presence of the tau protein in studies related to AD. The aggregation of the abnormal tau

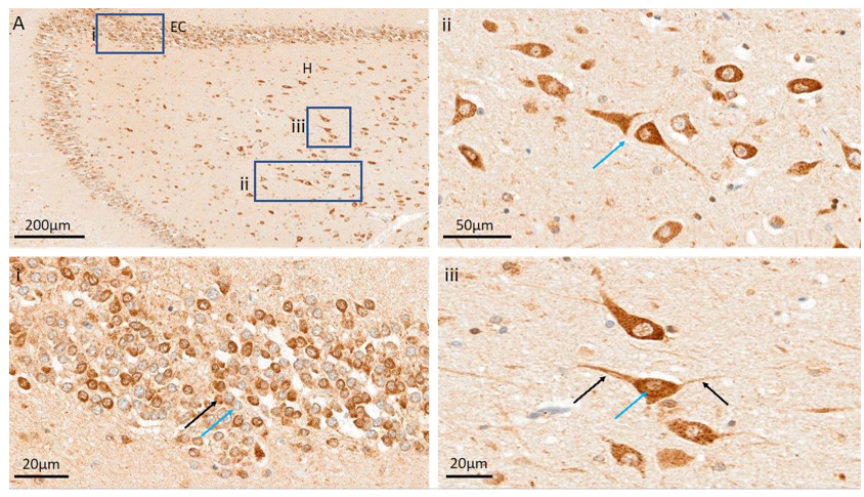

Figure 5: *A) Distribution patterns of C-menin in the hippocampus (H) and entorhinal cortex (EC) of the control human tissue. i) The outer layer of the hippocampus close to the EC exhibited the expression of C-menin in some neuronal population (black arrow), whereas other adjacent cell bodies were devoid of the signal (blue arrow). ii) Menin labeled processes were juxtaposed against another corresponding neuron's dendritic region (blue arrow) in the hippocampus. iii) Menin was expressed in both the cell body (blue arrow) as well as the axons and dendrites (black arrows) in the hippocampus.*

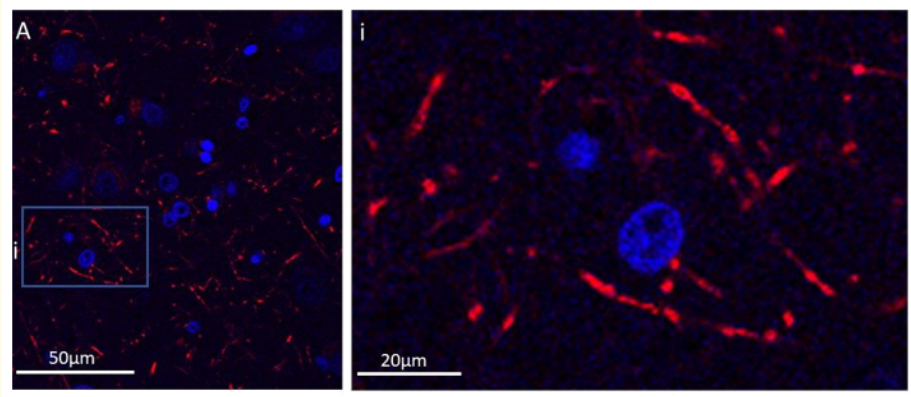

Figure 6: *A: Hippocampal slices were labeled with Alex555-conjugated Bungarotoxin and the cell body was labelled with DAPI in the control human hippocampus. i. The expression pattern of the α7 subunits of the nAChR were discernible in proximity of the cell body labeled blue.*

leads to the disruption of protein trafficking resulting in the perturbation of neuronal machinery, which in turn underlies AD pathology. As shown above, the slices were stained with optimized tau PHF antibody. Figure 7 shows a few patches of Tau-PHF in EC even though the

medical records of the control cases showed no signs of clinically reported dementia at the time of death. These PHF plaques were evident in layer III of the EC whereas as shown in figure 7 and the hippocampus region was devoid of any tau PHF plaques. These data suggest that indeed some plaques are evident in the "normal" control human brain. To ensure that there were significant differences between PHF plaque formation between control and AD brain, we then compared these brain regions with those where the tissue was obtained from individuals with a medical record of AD. Figure 8 demonstrates that tau expression is ubiquitous in an AD marked brain. Taken together, these data show that even in "normal aging brain" abnormal aggregates of tau are discernible but that this plaque formation is significantly more pronounced in an AD labeled brain [24].

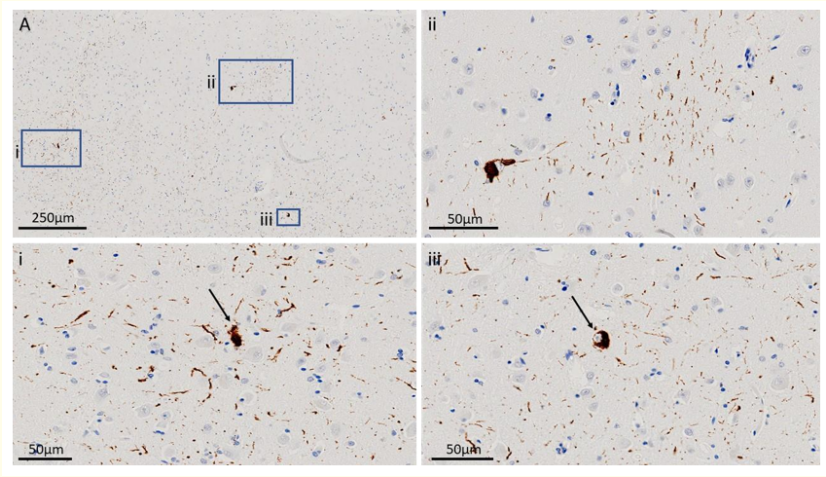

Figure 7: *A) Distribution patterns of tau in the top layer of the entorhinal cortex (EC) of control aging human tissue. i) Small fragments of tau accumulation, as pointed by the arrow, were evident in normal aging brain. ii) The fragmentation of tau protein was evident along with larger clumps. iii) The black arrow points to a cell body where the tau protein appears to have been accumulated intracellularly. All nuclei were stained with hematoxylin.*

Expression of C-menin in AD and ageing control hippocampus and entorhinal cortex

The data presented above demonstrates the presence of several synaptic proteins including C-menin in human tissue obtained from a normal aging brain. We next sought to determine their expression patterns in human brain tissues with established clinical history of dementia and compared them with their approximated age-matched controls.

For this study, ageing control and AD human cases as described in methods section were used to draw observations on the expression of C- menin, as mentioned earlier in the methods section. Figure 9 and 10 show the expression patterns of C-menin in hippocampus and the entorhinal cortex in both the old control and an AD brain respectively. These "clumps" have a close resemblance to the fragmented neuronal C-menin expression. This qualitative similarity of C-menin's abnormal expression in the aging brain, regardless of whether the tissue was inflicted with the AD disease or not is interesting. The C-menin expression in both the AD and the old control cases revealed fragmented pattern intracellularly where it appeared to have formed aggregates confined to the extracellular regions. It is important to

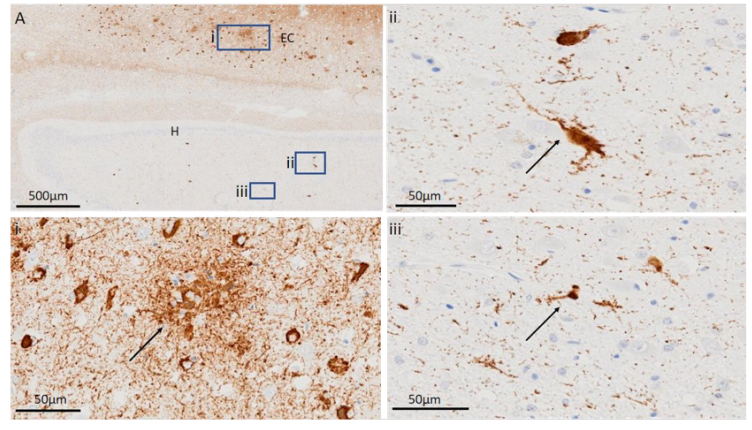

Figure 8: *A) Distribution patterns of tau in the hippocampus (H) and surrounding entorhinal cortex (EC) in an AD human tissue. i) The black arrow points to the aggregates of tau forming abnormally in the entorhinal cortical region. ii) The black arrow points to the fragmented/degenerated neuron with accumulated tau in the cell body and the neurites in the hippocampus. iii) The black arrow shows the accumulated tau protein in the neurites exhibiting fragmented processes in the hippocampus.*

note that many menin positive cells that were surround by Tau exhibited fragmented morphology and their cell bodies either appeared vacuolated or dead (Figure 10, see the insert ii and iii).

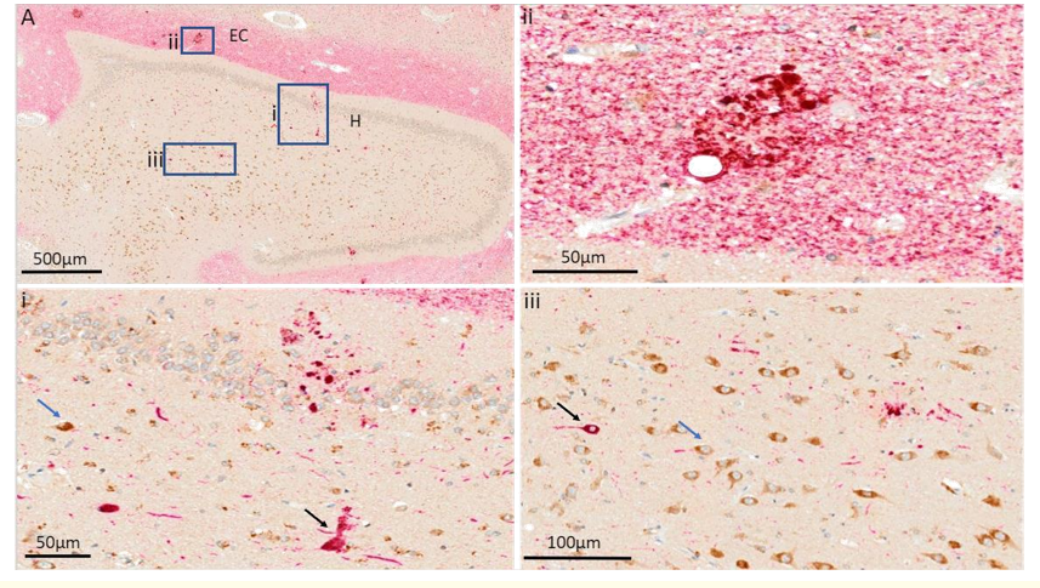

Figure 9: *A) Distribution patterns of tau (pink) and C-menin (brown) in the hippocampus (H) and surrounding entorhinal cortex (EC) of ageing control human tissue. i) The blue arrow indicates the presence of C-menin in the cell body, whereas the black arrow indicates the tau fragmentation occurring inside the hippocampus. ii) The presence of tau clumps is quite evident in the entorhinal cortex. iii) The black arrows point to a neuron where the tau is accumulating intracellularly and the blue arrow points to a neuron expressing C-menin in the hippocampus.*

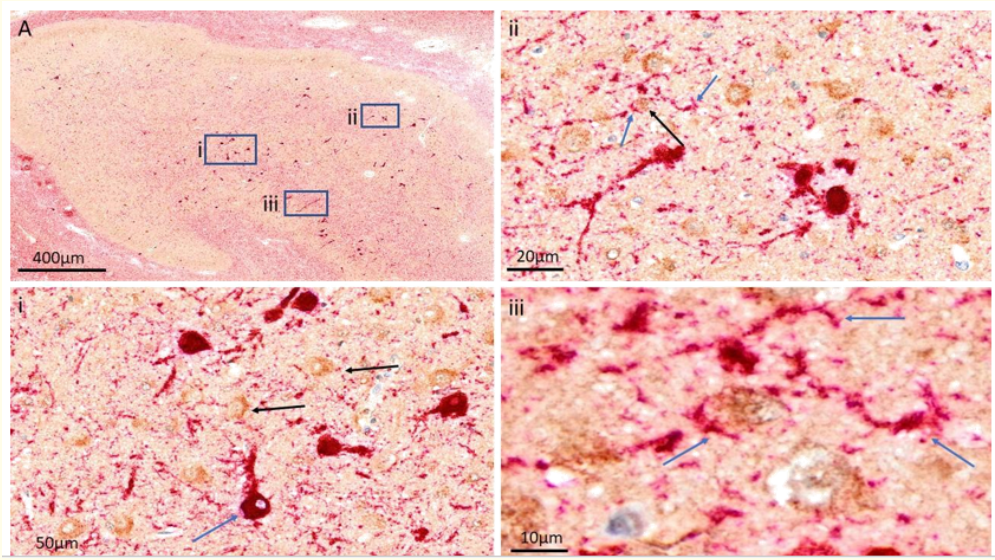

Figure 10: *A) The distribution patterns of tau (pink) and C-menin (brown) in the hippocampus of AD human tissue. i) The blue arrow shows a representative example of a neurons where extensive tau aggregates appear inside the somata and along the entire length of the neuron, whereas the black arrows indicate C-menin positive neurons. ii) Tau protein (blue arrows) appears to surround or accumulate around menin positive neurons (black arrow). iii) Extensive Tau accumulation surrounding menin positive neurons.*

Quantification of the C-menin, Tau and the extent of their co-localization in both controls and AD

To compare the relative expression of C-menin and tau across cases, the staining patterns were quantified using machine learning algorithm in QuPath software by training it to deduce the pixel classifiers blindly. To measure the extent of co-localization, the classifiers for DAB and MAG were applied sequentially and the co-localization between C-menin and tau was found to be much higher in the AD cases compared to their control counterparts in figure 11. In figure 12, we see that in the WB analysis there is an increase of menin to actin ratio in the AD cases compared to the controls.

Discussion

All present clinical strategies to treat Alzheimer's disease either pharmacologically targeting the acetylcholine receptors or neutralizing plaques have met with little to no success whereas the disease burden continues to increase. We believe that while the nicotinic acetylcholine receptors and Amyloid hypothesis are likely correct to a greater extend but the potential missing link in the breakdown of learning/memory and cognition circuits may occur at the level of trophic factor-depended, and activity-driven mechanisms involving genes such as *MEN1*. It is important to note that both the trophic factors and their receptors dysfunction [25-27] and the neuronal activity patterns are known to be compromised in AD [28-30]. Similarly, we have shown previously that the trophic factor mediated and activity-driven mechanisms in both invertebrate and vertebrates drive the expression of *MEN1* encoded protein menin, which in turn controls the expression and targeting of nAChRs [13,14]. Moreover, recent studies have shown that the conditional menin knockout in adult mice, renders their learning and memory dysfunctional [16]. Taken together with human data presented in this study, we propose

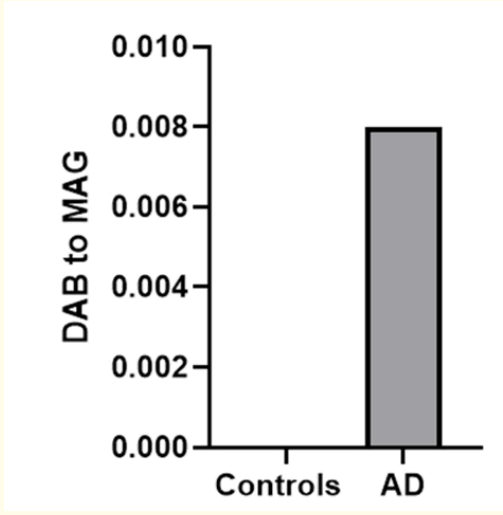

Figure 11: *A) The expression intensity of C-menin (DAB) and Tau (MAG) was quantified using the DAB and MAG classifier for all cases. The overlap between DAB and MAG was measured to determine the extent of co-localization between C-menin and tau and compared to the controls, the AD cases have a higher degree of co-localization.*

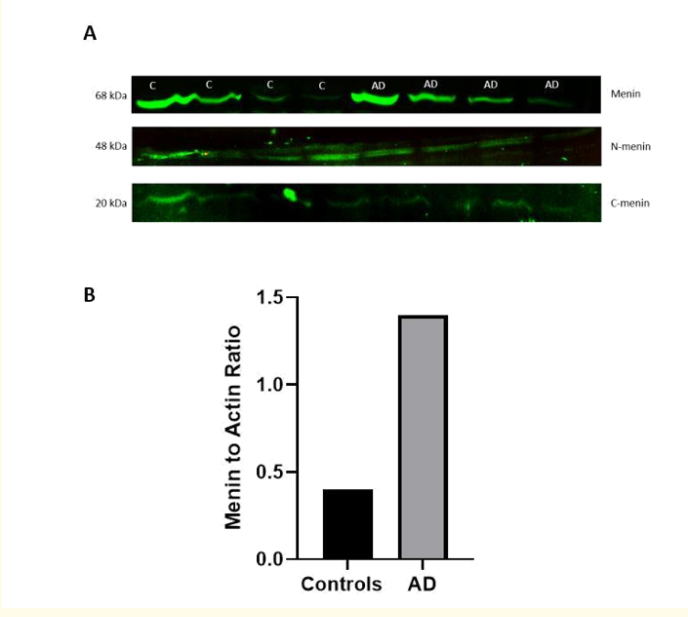

Figure 12: *Western blot results of full length menin to actin ratio show an increase in the menin expression in the AD cases compared to the controls. (A). Full length menin was present at its expected molecular weight of 68 kDa, N-menin at 48 kDa and C-menin at 20 kDa and was observed in both the AD and C. (B). Summary data, showing an increase in menin to actin ratio in the AD cases compared to the controls (n = 4 for controls and AD with equal sex distribution).*

a novel hypothesis that menin - a product of *MEN1* tumor suppressor gene, may regulate the expression of nicotinic acetylcholine receptors in the hippocampus in a trophic factor and activity-dependent manner. Moreover, we speculate that a perturbation of this cholinergic cascade in turn may lead to learning, memory and cognitive deficit seen in Alzheimer's patients. This study is the first to obtain significant preliminary evidence from human autopsied tissue of both Alzheimer's and non-Alzheimer's brains demonstrating the presence of menin in the hippocampus - the learning and memory center as well as the cortex. We have also demonstrated that menin expression appears to be compromised in the hippocampus, whereas cells exhibiting its immunoreactivity are either entrapped or invaded by Tau plaques in the diseased brains. These initial observations are novel, interesting and exciting but without additional molecular quantification of differential expression of menin in Alzheimer's brain, it will be too premature to establish an unequivocal link between *MEN1* and AD.

An extensive study integrating gene and protein expression derived from 414 clinically and neuropathologically confirmed AD patients has demonstrated that gene clusters corresponding to synaptic transmission, metabolism, cell cycle, survival and immune responses are indeed downregulated in tissues obtained from AD subjects. Importantly, a loss of Early Growth Response (EGR3) factor which causes synaptic deficit involving failed targeting of synaptic vesicles to their target sites may be an important contributing factor [31]. Furthermore, EGR3 which is a transcription factor and regulates synaptic activity is found to be essential for learning and memory in animal models [32]. EGR3-deficit mice were found to have profound defects in early and late phase hippocampal long-term potentiation (LTP) as well as short and long-term hippocampus and amygdala-dependent learning and memory [32]. These studies thus further demonstrate a link between growth factor response elements, synaptic proteins and deficits in LTP underlying learning and memory. Taken together, these data thus further underscore the importance of trophic molecules and their potential roles in learning and memory as seen in the AD. Furthermore, these studies also corroborate well with previously published role of menin in learning and memory [16] and its activation by trophic factor and activity dependent regulation of synaptic structures and function [14].

The paucity of morphological evidence for various synaptic proteins in the autopsied human brain owes its existence to the poor quality and integrity of the tissue obtained, and the inherent auto-fluorescence associated with the tissue. In this study, we first ensured that the tissue integrity was fully maintained, hence the evidence for this was sought through MAP staining. Moreover, instead of relying on fluorescent antibodies which usually generate tremendous false positives, we used an automated process, first to optimize the antibodies by using state of the art Dako Omnis system. This allowed us to obtain the highest degree of specificity with our non-fluorescent antibodies thus enabling double staining of two different proteins concurrently. The quality of our staining for all proteins labelled in this study is thus unparalleled in the literature. Whereas we did attempt to validate the differential expression patterns of these proteins molecularly, using western blot analysis, however, the compromised tissue integrity resulted in inconsistent outcomes. The freshly isolated tissue from autopsied brain will therefore be required to validate these otherwise morphological findings unequivocally.

Postsynaptic density-95 is an important scaffolding protein of the dendritic spines where it serves myriad functions ranging from anchoring membrane receptors in the spines, trafficking and localization of ion-channels and transmitter receptors in order to keep synaptic transmission in line with any given network's output. Perturbation of PSD-95 has been implicated in several synaptopathies, in psychiatric diseases as well as in dementia of AD type [33]. Similarly, amyloid accumulation in APP mutant mice neurons was shown to reduce PSD-95 and GluR1 levels [34]. However, studies on the role of PSD-95 in human brain are rare and its expression patterns have not been demonstrated before explicitly. It is interesting to note that in contrast with synaptophsin, we found PSD-95 to be localized in both neuronal somata, axons and dendritic projections suggesting that the synthesis and assembly of these proteins may be ubiquitous, whereas the former may only be synthesized in the extra-somal compartments invoking local protein synthesis machinery.

Synaptophysin on the other hand, is an integral membrane protein of small synaptic vesicles distributed throughout the nervous system - comprising almost 90% of vesicles where it may serve functions such as calcium binding, vesicle recycling, endocytosis etc. Its dysfunction has been implicated in neurodevelopmental disorders such as schizophrenia [35-37]. Western blot and immunohistochem-

istry conducted on autopsied human tissue obtained from various ages also demonstrated a pattern of localization as has been demonstrated present in our study [38]. Specifically, these authors demonstrated that whereas synaptic density increased steadily from birth to late childhood followed by a decrease in early adolescence to reach adult levels by late adolescence. Although the pattern of expression of synaptophysin observed in this study was similar to that of the primate, it did not examine tissue obtained from AD brains [38]. Spatio-temporal and developmental expression patterns of PSD-95, synaptophysin, nAChRs and menin have recently been demonstrate in a mouse model and the data corresponds well with the human study [15]. It is also interesting to note that PSD-95 levels decrease after the loss of synaptophysin, and these steps preceded the onset of AD in the order of: 1) α7 deposition, 2) presynaptic disruption, 3) postsynaptic disruption coupled with phosphatidylcholine reduction and 4) the neuronal loss [39]. The lack of synaptophysin staining in the somata and its initial axon segments was surprising, and it raises two issues. On the one hand, it is possible that the synaptophysin may either be synthesized *de novo* - in the extra-somal compartments [40,41] or on the other hand, its soma to axonal transport may be perturbed in degenerative diseases or in an aging brain [42]. These postulates, however, would need to be tested experimentally and may only be feasible in an animal model.

The hippocampal formation is the main target of cholinergic axonal projecting from the medial septum (MS) and diagonal band of Broca (DBB) along the septo-hippocampal pathway, which terminate on both pre- and post-synaptic structures of glutamatergic and GABAergic neurons throughout the hippocampus. Activation of hippocampal nAChR is known to regulate the expression of a variety of NTF which promote synapse formation and plasticity, and also the survival, maturation and synaptic integration of adult-born neurons. It is also interesting to note that - amyloid peptide has been shown to block the responses of α7-containing nicotinic receptors in hippocampal neurons [8]. Taken together, and consistent with the notion that impaired cholinergic transmission and a significant reduction in the number of nicotinic binding sites are the hallmark of AD [43] it stands to reason that nAChRs function may be compromised by proteins such as Tau. Consistent with this postulate are the studies that have shown that neurons with accumulated HP-Tau in human cerebral cortex expressed either little or no α4 and α7 nAChRs mRNA as compared with the adjacent neurons that were devoid of Tau. These data thus suggest that Tau accumulation may impair nAChRs expression at the mRNA level [43] however the mechanisms underlying this reduction of receptor expression remain unknown. Based on our observations presented in figure 9 and 10, we propose that Tau either directly or indirectly may perturb menin expression or function thus rendering dysfunctional the nAChRs mRNA or the receptor targeting to specific synaptic sites. This postulate would, however, needs to be tested experimentally.

The data presented in 11 shows different expression patterns of C-menin across all cases. It is interesting to note the extent of co-localization between the tau protein and C-menin in the AD cases, which suggests that the interaction between tau and C-menin could potentially underlie the perturbation of the alpha 7 subunit, this however would need to be further investigated and determined experimentally. We have also shown here that the expression of full length menin decreases at the protein level in the human hippocampi for AD compared to the controls cases (Figure 12).

Conclusion

Taken together, the present study provides first direct evidence for the presence of menin in human brain tissue obtained from both AD and non-AD samples. Moreover, we have shown that menin is not only present in the cytoplasmic regions but also throughout the neuronal process and also at putative synaptic sites - consistent with our recently published study in a mouse model [15]. We have also demonstrated the expression patterns of other synaptic proteins and the nAChRs in a human tissue at a resolution never shown before. The data demonstrates for the first time that menin and tau protein either co-localize or are in close proximity to each other in tissues obtained from AD brain. Further studies are however, required to provide qualitative and molecular evidence for menin expression levels and its interactions with Tau and other markers of AD.

Supplementary Data (Appendix I)

Cases	Neuropathological Findings	Cerebral Amyloid Angiopathy (CAA)	Minor Neuropathological Findings
BB19-015 block B27; Young Alzheimer: 73 woman	High Level of Alzheimer Neuropathologic Change 1. Amyloid phase (A; Thal): 5 out of 5 2. Tau stage (B; Braak and Braak): 6 out of 6 3. Neuritic amyloid plaque (C; CERAD): 3 out of 3 4. National Institute on Aging - Alzheimer's Association (NIAAA) ABC score: A3B3C3	A. Thal CAA Stage: 2 (allocortical) B. Love CAA Score: 3 (widespread circumferential) C. Capillary CAA: present D. CAA vasculopathy: widespread (vascular "cracking") E. Microvascular lesions: absent F. Leukoaraiosis: moderate	Cerebrovascular Microvasculopathy A. Arteriolosclerosis (arterioles): mild B. Venous collagenosis: moderate C. Artery and arteriole mineralization: absent D. Thal staging (Thal, 2003): Stage A: basal ganglia and deep cerebral white matter E. Severity of secondary changes: not applicable (see cerebral amyloid angiopathy) Terminal Ischemic Encephalopathy
BB16-012 block B24; Old Alzheimer: 90 man	I. Alzheimer's Disease, moderately severe, right hippocampus more severe than left A. High Level of Alzheimer Neuropathologic Change 1. Amyloid phase (A; Thal): 4 out of 5 2. Tau stage (B; Braak and Braak): 6 out of 6 3. Neuritic amyloid plaque (C; CERAD): 3 out of 3 4. National Institute on Aging - Alzheimer's Association (NIAAA) ABC score: A3B3C3 B. Distribution 1. Neocortex a. Frontal lobe: moderate to marked b. Temporal lobe: moderate to marked c. Parietal lobe: moderate to marked d. Occipital lobe: 2. Allocortex and corticoid regions a. Entorhinal cortex: moderate to marked b. Amygdala: marked	A. Thal CAA 1 (neocortical) B. Love CAA Score: 1 (scant) C. Capillary CAA: absent D. CAA vasculopathy: absent	Microscopic focus of putamen ischemia, remote Myelin pallor, gracilis column in cervical spinal cord, consistent with length-dependent neuropathy

	c. Hippocampus: moderate (left); marked (right) 3. Diffuse Systems a. Basal nucleus of Meynert (acetylcholine): neuron loss - moderate; neurofibrillary tangles - frequent b. Locus ceruleus (norepinephrine): neuron loss - mild; neurofibrillary tangles - frequent c. Raphe nuclei (serotonin): neuron loss - sparse; neurofibrillary tangles - frequent		
BB17-006 block B24; Young Control: 73 man	NA	NA	Normal brain (weight: 1652 grams) Minimal agonal changes
BB15-012, block B2; Old Control: 93 woman	NA	NA	Low Level of Alzheimer Neuropathologic Change National Institute on Aging – Alzheimer's Association ABC score = 1,1,2 - A - Thal amyloid phase: 1 - B - Braak tau stage: 3 to 4 - C - CERAD neuritic plaques score: 1 (neocortex) Beta-amyloid Congophilic Angiopathy, Vonsattel grade 2 out of 4 Cerebrovascular Microangiopathy, mild - Arteriolosclerosis, moderate - Leukoaraiosis: mild - Perivascular parenchymal rarefaction: mild Early terminal ischemic encephalopathy

Cases for western blot: The hippocampal sections frozen contained the dentate gyrus, CA3, CA2, and CA1 regions, and the end of the subiculum. The scores are the ABC scores from the NIA-AA scheme. The cognitively normal people have some neocortical beta amyloid. A0 indicates no neocortical amyloid and A3 indicates extensive presence of neocortical amyloid.

Cases	Age	Sex	Scores
BB21-004	69	Male	AD-A1B0C0
BB19-013	75	Male	AD-A1B1C0
BB18-012	78	Female	AD-A0B0C0
BB18-004	60	Male	AD-A0B1C0
BB18-003	71	Male	AD-A3B3C3
BB18-005	90	Female	AD-A3B3C3
BB18-016	71	Male	AD-A3B3C3
BB20-009	59	Female	AD-A3B3C3

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Volume 15 Issue 5 May 2023