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Biochemical analyses of tau and other neuronal markers in the submandibular gland and frontal cortex across stages of Alzheimer disease

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Abstract

Hyperphosphorylation of the microtubule-associated protein tau is hypothesized to lead to the development of neurofibrillary tangles in select brain regions during normal aging and in Alzheimer disease (AD). The distribution of neurofibrillary tangles is staged by its involvement starting in the transentorhinal regions of the brain and in final stages progress to neocortices. However, it has also been determined neurofibrillary tangles can extend into the spinal cord and select tau species are found in peripheral tissues and this may be depended on AD disease stage. To further understand the relationships of peripheral tissues to AD, we utilized biochemical methods to evaluate protein levels of total tau and phosphorylated tau (p-tau) as well as other neuronal proteins (i.e., tyrosine hydroxylase (TH), neurofilament heavy chain (NF-H), and microtubule-associated protein 2 (MAP2)) in the submandibular gland and frontal cortex of human cases across different clinicopathological stages of AD (n = 3 criteria not met or low, n = 6

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CRediT authorship contribution statement

Yamah Hamsafar: Data curation, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. **Qian Chen:** Formal analysis, Methodology, Writing – review & editing. **Alexander D. Borowsky:** Methodology, Resources, Writing – review & editing. **Thomas G. Beach:** Conceptualization, Data curation, Funding acquisition, Resources, Writing – review & editing. **Geidy E. Serrano:** Data curation, Writing – review & editing. **Lucia I. Sue:** Data curation, Writing – review & editing. **Charles H. Adler:** Data curation, Funding acquisition, Writing – review & editing. **Douglas G. Walker:** Methodology, Writing – review & editing. **Brittany N. Dugger:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2023.137330.

intermediate, and n = 9 high likelihood that dementia is due to AD based on National Institute on Aging-Reagan criteria). We report differential protein levels based on the stage of AD, anatomic specific tau species, as well as differences in TH and NF-H. In addition, exploratory findings were made of the high molecular weight tau species big tau that is unique to peripheral tissues. Although sample sizes were small, these findings are, to our knowledge, the first comparison of these specific protein changes in these tissues.

Keywords

Submandibular gland; Big tau; 4a exon; Tau; Phosphorylated tau (p-tau); Tyrosine hydroxylase; Neurofilament heavy chain; Microtubule-associated protein 2

1. Introduction

Tau proteins are a class of microtubule-associate proteins that promote the polymerization and stabilization of microtubules in neuronal cells, and glial cells to a lesser degree [1]. It is evident phosphorylated tau (p-tau) is the main component of neurofibrillary tangles (NFTs) which are implicated in Alzheimer disease (AD) [2–4]; a disease staged by the involvement of NFTs starting in the transentorhinal region of the brain and proceeding into other cortical areas such as frontal cortex regions [5,6]. Although tau and its isoforms have been studied extensively in the cerebrum, few studies investigate involvement within the peripheral nervous system in human cases [7–16].

Our previous studies have examined the relative distribution of p-tau in the human brain and spinal cord in AD and non-demented (ND) cases and revealed spinal cord tau pathology in over 95% of persons with AD and 50% of elderly ND persons [17]. Additionally, the cervical cord region, most proximal to the brain and brainstem, contained the highest frequency of p-tau deposits [17]. This suggests phosphorylation of spinal cord tau proteins may occur in preclinical stages of AD, and although this conventionally is thought to start in the transentorhinal region, it is also possible that brainstem regions might be amongst the first affected, facilitating early spread to the cord with a cranial-caudal sequence. Thus, the concept of neuroanatomical spread of tau pathology could be extended to include not only the brain but also the spinal cord. Whether tau pathology, like alpha-synuclein pathology [18,19], also eventually spreads to the peripheral nervous system is yet to be determined. A follow up study selected the submandibular gland as a site for the investigation of tau species in the peripheral nervous system because it demonstrated the highest total tau level in an extensive set of peripheral tissues (i.e., sigmoid colon, scalp, abdominal skin, liver, and submandibular gland) [20]— reported as 1.72% of the tau protein levels found in the frontal cortex by enzyme linked immunosorbent assays (ELISA). Additionally, previous related studies have determined the feasibility of submandibular gland needle biopsies for diagnoses of select neurodegenerative diseases [21–24,46].

Other studies have discussed the structure of tau in the peripheral nervous system, with focus on a high molecular weight tau species, containing a 4a exon, commonly denoted as big tau [16,25–29] — given its 110 kDa molecular weight, whereas the unmodified conventional six brain isoforms, with combinations of either 0N, 1N, or 2N and 3R or 4R

based on the alternative splicing of the *Microtubule-Associated Protein Tau (MAPT)* gene, have apparent molecular weights ranging from 45 to 65 kDa [2]. Investigations into big tau have been confirmed in but mainly restricted to rodent models, to our knowledge; however, one previous study attempted to confirm its presence in the enteric nervous system in human cases, with no success [16]. Although big tau's function is not implicitly understood, understanding its distribution and levels across different disease stages will set the stage for further investigations. In this study, we used biochemical methods to comparatively investigate multiple tau species (using HT7 and T231 antibodies for unmodified tau and p-tau phosphorylated at T231, respectively) and other neuronal proteins i.e., tyrosine hydroxylase (TH), neurofilament heavy chain (NF-H), and microtubule-associated protein 2 (MAP2) across different clinicopathological stages of AD in the submandibular gland and frontal cortices to provide insights into selective vulnerability of the disease.

2. Materials and methods

2.1. Case selection

This study utilized samples collected from autopsied cases in the Arizona Study of Aging and Neurodegenerative Disorders (AZSAND) of Banner Sun Health Research Institute, and its Brain and Body Donation Program (BBDP) (website: https://www.brainandbodydonationprogram.org) [30,31]. The BBDP is focused on longitudinal clinicopathological studies of normal aging, dementia, and parkinsonism, and all participants, next of kin, or their legal representatives gave informed consent for annual clinical research assessments, autopsy, and tissue donation [31]. The database was queried for cases with an AD clinicopathological diagnoses, defined by the National Institute on Aging-Reagan (NIA-Reagan) criteria [32], and were further classified as criteria not met/low, intermediate, or high likelihood of AD. In addition, cases lacked a concurrent clinicopathologic diagnosis of other diseases including: vascular dementia, Parkinson's disease, dementia with Lewy bodies, frontotemporal dementia, hippocampal sclerosis, progressive supranuclear palsy, and corticobasal degeneration. We focused on select anatomical regions i.e., submandibular gland and frontal cortex based on previous investigations [20,21]. A total of eighteen cases having available submandibular gland and frontal cortex samples and meeting inclusion criteria were used; n = 3 criteria not met/low, n = 6 intermediate, and n = 9 high likelihood of AD. The demographics of the 18 cases in this study including age at death, postmortem interval (PMI), and gender for each group are listed in Table 1. There are no significant differences between different stages of AD with respect to age at death, gender, or PMI.

2.2. Tissue preparation and Western blots

Tissue processing methods have been previously described in related studies [33,34]. In comparison to previous studies of select tau species in the submandibular gland wherein tissues were processed with formic acid [20], this study uses RIPA buffer supplemented with SDS for protein extraction, a much milder detergent for extraction of soluble tau species as well as the additional neuronal proteins examined [34]. In brief, frozen tissue samples from each region were dissected on dry ice with a sterile scalpel and weighted; 40 mg for the submandibular gland, and 100 mg for the frontal cortex. Samples were then

lysed in 400–600 μl pre-cooled radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1.0 mM EDTA, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, pH 7.6; Thermo Scientific™, Waltham, MA, USA cat. 89900) supplemented with a protease and phosphatase inhibitor cocktail mix (Thermo Scientific™, cat. 78440). After briefly vortexing and incubating on ice for 10 min with agitation, the samples were sonicated in ten 5-second interval strikes on ice, with a 20-second cooldown period between strikes to prevent the samples from overheating. Samples were then briefly vortexed and centrifuged at 21,130 RCF (× g) for 20 min at 4 °C. The supernatant was aliquoted and stored at −80 °C for biochemical analysis. To determine total protein concentration, the supernatant was diluted at 1:10 for use in a bicinchoninic acid assay.

In Western blot assays, 20 μg of total protein from submandibular gland and 1.0 μg from frontal cortex samples were used to investigate protein levels. Samples were treated with 4 × Laemmli sample buffer (BioRad, Hercules, CA, USA cat. 1610747) supplemented with 2-Mercaptoethanol (BioRad, cat. 1610710) and then heated for 10 min at 80 °C per manufacturer recommendations. Samples were subsequently cooled on ice, then loaded onto NuPAGE bis-tris 4–12% stacking gels (Invitrogen, Waltham, MA, USA cat. NP0323) with protein ladders (BioRad, cat. 1610375) and tau protein ladders of all six isoforms (rPeptide, Watkinsville, GA, USA, cat. T-1007–1). Gels were then subjected to gel electrophoresis in reduced conditions for approximately two hours at 110 V, and then subsequently moved to a wet-transfer module for one hour at 30 V to transfer onto a nitrocellulose membrane (Invitrogen, cat. LC2001). The following steps in this section proceed with gentle agitation on a rocker; the nitrocellulose membrane was gently rinsed in phosphate-buffered saline (PBS) buffer solution for 10 min and then blocked with a buffer comprised of 5% w/v nonfat powdered milk in PBS for one hour. The membrane was then probed with primary antibodies diluted in a buffer comprised of 5% w/v nonfat powdered milk in PBS with 0.05% Tween-20 (PBS-T (0.05%)) overnight at 4 °C. After incubation, the primary antibody solution was decanted, and the membrane rinsed three times for 10 min each in PBS-T (0.05%) before being probed with secondary antibodies diluted in 5% w/v nonfat powdered milk in PBS-T (0.05%) for one hour at room temperature. After a final membrane rinse three times for 10 min each in PBS-T (0.05%) to decant the secondary antibody, the membranes were imaged on a Licor Odyssey CLx (Li-COR Biosciences, Lincoln, Nebraska, USA) using auto exposure settings for the 680 nm and 800 nm channels. Western blot signal intensities were quantified with the ImageStudio Lite™ software (Li-COR Biosciences) to evaluate protein levels. The primary and secondary antibodies used to evaluate investigated proteins are listed in Tables 2 and 3, respectively.

Areas for quantifications were selected based on the Western blot areas producing the largest signal intensities and were inclusive of areas proximal to know the banding patterns for the epitope. In addition, corresponding ranges of the tau ladder were also used as references. In the case of tau, bands were analyzed with a large signal intensity relative to backgrounds and proximal to areas denoted by the isoform ladder. NF-H was quantified only in the upper band between the 150 kDa and 250 kDa marker that was present in both western blots. As there were numerous bands of MAP2 in frontal cortex (per manufacturer notes there are observed band sizes of 280 and 70 kDa in mouse brain lysates), a larger area spanning at ~250 kDa to ~70 kDa was evaluated, while only a single prominent band was present

in submandibular gland and quantified. For TH, per datasheet from the manufacturer, the expected band was at 58 kDa; as there were similar band near that region that is what we evaluate for the submandibular gland, no band were present for the frontal cortex samples.

2.3. Statistical analysis

Quantitative analysis methods done in this study for measuring signal intensities in Western blot assays have been previously described [35,36]. Western blot signal intensities measured with ImageStudio Lite™ were normalized with controls across each gel for variation in antibody binding efficiency, blocking, washing, and other procedural steps. In addition, all Western blot assays were run in triplicates, and their quantitative results were averaged across each run to determine a final relative protein level for tau and other neuronal proteins in each case. To compare data across different stages of AD, cases were categorized into data sets based on their AD clinicopathological status, defined by NIA-Regan criteria (criteria not met/low, intermediate, and high likelihood) [32]. In each data set, the mean and standard deviation of the protein levels were calculated and used in a two-sample *t*-test with equal variance to determine if there were significant differences across different stages of AD for: (1) low vs. intermediate, (2) low vs. high, and finally (3) intermediate vs. high likelihood. Due to low sample sizes, data were not adjusted for age at death, gender, or other demographic variables. T-tests were evaluated with a level of significance of $p = 0.05$.

3. Results

3.1. Qualitative observations of tau and P-Tau in the submandibular gland

Western blots for tau (HT7) and p-tau (T231) in the submandibular gland are presented in Fig. 1.A., with tau protein ladder and frontal cortex samples for comparison. The tau protein ladder presented six bands, corresponding to the six tau isoforms, with a molecular weight range of approximately 48–56 kDa (Fig. 1.A. far right of the diagram and supplemental figures). The bands are labeled based on the expected molecular weight for each isoform as well as manufacturer information. The lower five bands are presented alongside the submandibular gland experimental samples in Fig. 1.A. Tau Western blots in this region presented several bands within the same molecular weight range of 48–56 kDa (Fig. 1.A., tau), as well as one or two additional higher molecular weight bands at approximately 75 kDa or 75 and 80 kDa, respectively. In our supplemental Fig. 1 we present an expanded view of a tau Western blot in the submandibular gland, and demonstrated that there were limited/faint banding patterns In the 110 kDa range with this antibody. In comparison to the 4a exon specific antibody (Big tau (4a) Fig. 3) there were similar banding patterns as with the tau antibody, but with exceptional dark bands across all submandibular gland samples at 110 kDa that was lacking in frontal cortex samples. P-tau Western blots presented several bands, albeit with limited separation and distinction between them, within the molecular weight range of approximately 50–58 kDa (Fig. 1.A., P-tau). P-tau Western blots presented a faint band with a molecular weight of approximately 110 kDa.

3.2. Qualitative observations of tau and P-Tau in the frontal cortex

Western blots for tau and p-tau in the frontal cortex are presented in Fig. 2.A. Tau Western blots presented four distinct bands across all cases examined in this study, with

molecular weights of approximately 48, 50, 51, and 53 kDa. In one intermediate and five high likelihood of AD cases there were additional bands, with molecular weights of approximately 54 and 56 kDa. We hypothesize these six identified bands correspond to the 0N3R, 0N4R, 1N3R, 1N4R, 2N3R, and 2N4R tau isoforms, respectively, and are therefore identified to the right of Fig. 2.A. Additional data on these specific bands including statistical analyses are included in the supplemental table and supplemental Fig. 2. P-tau Western blots also presented four distinct bands across all cases in this study, with molecular weights of approximately 50–54 kDa. In only seven high likelihood cases, there is an additional one to two present and distinct bands with molecular weights of approximately 56 and 58 kDa. As with tau Western blots, these p-tau bands are identified to the right of Fig. 2.A. with their corresponding tau isoform label.

3.3. Quantification and statistical analysis of tau and P-tau Western blots across clinicopathologic diagnoses in each region

The areas used to measure signal intensities in each Western blot are exemplified with dashed boxes (Fig. 1.A. and 2.A.). The mean and standard deviation for protein levels in each data set (i.e., cases with a criteria not met/ low, intermediate, or high likelihood AD contributed to dementia) are presented as cluster bar graphs (Figs. 1.B. and 2.B. for the submandibular gland (20 μg) and frontal cortex (1 μg), respectively). Tables 4 and 5 present the results of the two-sample *t*-test with equal variance to determine significant differences in protein levels of tau and other neuronal proteins across different stages of AD for: (1) criteria not met/low vs. intermediate, (2) criteria not met/low vs. high, and finally (3) intermediate vs. high likelihood for each of these regions. In the submandibular gland sample set, there is a statistically significant increase in tau protein levels in intermediate and high vs. low likelihood ($p = 0.004$ and 0.042, respectively) (Fig. 1.B., Table 4). P-tau presented increased protein levels with the progression of AD but demonstrated no statistically significant differences (Fig. 1.B., Table 4). Our data does suggest an increase in the P-tau (110 kDa band) in high vs. low likelihood ($p = 0.048$), but this still may be due to chance if a stricter alpha value was applied.

In the frontal cortex, there was a significant decrease in tau protein levels in high vs. low likelihood ($p = 0.002$) (Fig. 2.B., Tau, Table 5). With respect to P-tau, there was a significant increase in detected protein levels in high vs. intermediate likelihood ($p = 0.036$) (Fig. 2.B., Table 5), and which may be attributed to the appearance of the high molecular weight bands present in cases with high-likelihood of AD as previously denoted.

3.4. Big tau species

To provide additional evidence of big tau species, one hypothesized to be specific to the periphery we utilized a tau 4a exon specific polyclonal antibody (courtesy of Drs. Itzhak Fisher and Peter Baas at Drexel University College of Medicine, Philadelphia, PA) on a subset of 9 submandibular gland samples across AD disease stage; frontal cortex of an AD and non-demented individual were used as controls [28,37]. The antibody was developed via bacterial fusion with the mouse 4a tau sequence and while this sequence is not highly conserved across mouse species, there is at least a fifty percent overlap and experimentally we did achieve some reactivity to present (Fig. 3). Utilizing our processed

samples and standard western blot procedures outlined above, we detected bands only within submandibular gland, at approximately 110 kDa, as well as other additional bands spanning from approximately 75 kDa to 50 kDa perhaps representing different tau isoforms; no bands were detected within frontal cortex (Fig. 3).

3.5. Other neuronal proteins i.e., TH, NF-H, and MAP2

TH was observed only in the submandibular gland, whereas NF-H and MAP2 were observed in both the submandibular gland and frontal cortex. Western blots of TH presented a distinct band in the submandibular gland with a molecular weight of approximately 58 kDa — its expected molecular weight, and additional fainter bands slightly below. TH was not detected in the frontal cortex, even after adjusting methods i. e., increasing the total amount of protein loaded to 20 μg or exposure time. In the submandibular gland, TH protein levels displayed a decrease in intermediate and high vs. low likelihood, with the lowest levels in intermediate likelihood (Fig. 1.C., Table 4). In the submandibular gland, NF-H Western blots presented a distinct band with a molecular weight of 210 kDa in both regions— its expected molecular weight (Fig. 1.A. and Fig. 2.A.), and in the frontal cortex this band was also present and an additional faint band at approximately 150–140 kDa (Fig. 2.A.). In the submandibular gland, there was a significant decrease in NF-H when comparing intermediate vs. low likelihood ($p = 0.017$) (Fig. 1.B., Table 4). In both regions, NF-H protein levels generally decreased with an increasing clinicopathological stage of AD. MAP2 Western blots presented different patterns in the submandibular gland than in the frontal cortex, however, it appeared neither region displayed results that corresponded to the expected molecular weight of 70 kDa (MAP2c isoform) or 280 kDa (MAP2a and MAP2b isoforms)— per manufacturer descriptions. In the submandibular gland, the MAP2 protein presented as a single distinct band at 110 kDa across all cases (Fig. 1A., MAP2). In the frontal cortex, the MAP2 protein presented multiple banding patterns, including two distinct bands at approximately 150 kDa and 200 kDa across few cases, however, these were accompanied by many non-specific banding patterns ranging from 135 to 250 kDa (Fig. 2.A., MAP2) and were only present after increasing the total amount of protein loaded to 20 μg. No trends were observed in the submandibular gland; in the frontal cortex, MAP2 protein levels generally decreased with an increasing clinicopathological stage of AD but this was not statistically significant (Fig. 2.B., Table 5).

4. Discussion

The current study, although sample size is small and methods are limited, presents additional evidence that tau and p-tau are detectable in the submandibular gland, and select protein levels change across distinct clinicopathological stages of AD. In addition, we provide additional evidence, and the first of our knowledge to demonstrate using a specific 4a exon antibody, high molecular weight tau (with molecular weight of 110 kDa), commonly referred to as big tau, present within the submandibular gland.

This study confirms previous reports of the relative protein levels of tau within the submandibular gland in comparison to the frontal cortex. The approximate percentage of tau in these peripheral tissues are within a 1.0–3.2 % range relative to the frontal cortex

based on clinicopathological stage of AD [17]— although it should be noted methods in tissue processing varied compared to the previous studies as the current study examined more soluble protein species. Furthermore, p-tau protein levels in the submandibular gland were 6.5–13.4 % relative to the frontal cortex based on the stage of AD. This allows us to understand scale of this work and appreciate the peripheral nervous system as a conjugate site to understand and develop insight for future works as these systems contain the same hallmark pathological proteins to a certain and worthwhile extent. Examining more soluble protein species, we demonstrate select tau species to increase in the submandibular gland as AD stage increased, while other neuronal proteins, NF-H decreased. With respect to the frontal cortex, total tau species decreased as well as there were trends with NF-H and MAP2, and there was an increase in P-tau species. It is likely the decrease seen in the frontal cortex is attributed to neuronal loss, however, it is unclear what to attribute the increase in tau levels to in the submandibular gland.

With respect to specific peripheral tau species, we did detect a high molecular weight band at approximately 110KDa with p-tau, and a weak/faint signal intensity with tau (HT7) at that molecular weight (supplemental Fig. 1, Fig. 1). To provide additional evidence that this is a big tau species, we received tau 4a exon specific polyclonal antibodies (courtesy of Drs. Itzhak Fisher and Peter Baas at Drexel University College of Medicine, Philadelphia, PA) [37]. This is the most specific antibody available, to our knowledge, to big tau. We detected bands only within submandibular gland and not within frontal cortex (Fig. 3). This is the first study to our knowledge to utilize a specific big tau antibody and detect it in submandibular gland tissues of humans. In addition to this finding, it may be noteworthy, yet highly speculative, to note in tau Western blots in the submandibular gland there were distinct bands at 75 kDa as well. There are many possibilities as to the species of this band, as it may be related to alternative splicing, post-translational modification, and/or any other process i.e. non-specific reactivity [38,39]. Additional biochemical applications in conjunction to gel electrophoresis and excretion of proteins from gels there may be possibility to determine the amino acid composition by protein chemistry/proteomics.

Other neuronal proteins: TH, NF-H, and MAP2 were additionally evaluated to provide insight into the selective vulnerability of AD and expand understanding of the disease mode i.e., if changes may be tau specific or span to other neuronal proteins. Previous studies have suggested that in the progression of AD there may be selective impairment of submandibular gland function i.e., a diminished salivary fluid volume [7]. Sympathetic innervations and a subset of neuronal cells within the parasympathetic submandibular ganglion which innervate the submandibular gland express TH [40,41], the rate-limiting enzyme of the catecholamine pathway, therefore, we were determined to understand if there are discernable changes of TH protein levels across different stages of AD. TH protein levels decreased in the submandibular gland, however, this change would implicate excessive salivation such as in cases with TH deficiency syndrome [42]. MAP2 and NF-H were selected as structural neuronal markers. MAP2 is a neuron-specific protein that stabilizes microtubules in the dendrites of cells [44,45]. NF-H is a type IV intermediate filament, one protein in a family of structural proteins (i. e., neurofilament light and medium chains) providing support for axons [45]. Similar studies have investigated neurofilament light (NF-L), medium (NF-M), and heavy (NF-H) chains as nonspecific biomarker of axonal injury and an *in-vivo* fluid

biomarker of neurodegenerative diseases [43]. In both regions, NF-H protein levels generally decreased with an increasing clinicopathological stage of AD and in the frontal cortex only MAP2 generally decreased- although there was high variability in the data. The decrease in neuronal protein levels may suggest an overall loss of neuronal innervations in either region in response to the progression of AD. Additional research on the neurotransmitter system such as with immunohistochemistry sampling throughout the submandibular gland is warranted to make clear conclusions about decreases in innervations.

This study had multiple limitations. As this study involved human tissues and of a specific area (submandibular gland) limited quantities and type were available at the time of experimentations. As we had limited frozen samples, we focused on western blot analysis in these exploratory analyses. Further works, when resources and additional samples have amassed will include additional samples specifically those within the criteria not met/low likelihood of AD category to understand variabilities, as well as methodologies (such as immunohistochemistry and ELISAs). Our sample size was small, with only n = 3 criteria not met/low, n = 6 intermediate, and n = 9 high likelihood of AD. A more even distribution of cases is recommended for future studies as well as additional studies investigating sampling procedures- such as rostral/caudal, medial/lateral, and inferior/superior aspects of regions. For methodologies, additional works with immunohistochemistry can aid in cellular/structural localization/co-localizations of these proteins, and ELISA analyses can provide additional means of quantitation, however, it may be difficult to obtain given the number of controls and/or low disease cases available within biorepositories.

5. Conclusion

This study is the first to our knowledge to investigated tau and other neuronal proteins in both the submandibular gland and frontal cortex of human cases across different clinicopathological stages of AD. We demonstrated via biochemical analysis there are unique changes based on anatomical region. These findings are explorative and emphasize the importance of investigating peripheral tissue in AD and related dementias. In addition, these findings set the stage for understanding the selective vulnerability of AD in the peripheral nervous system by implicating changes of certain other neuronal proteins such as TH and NF-H, and to some extent MAP2. Finally, the identification of high molecular weight tau isoforms, such as big tau, in the submandibular gland in human cases is to our knowledge one of the first instances confirming its involvement in this peripheral tissue, and additional studies are warranted given this finding to further investigate its role in health and disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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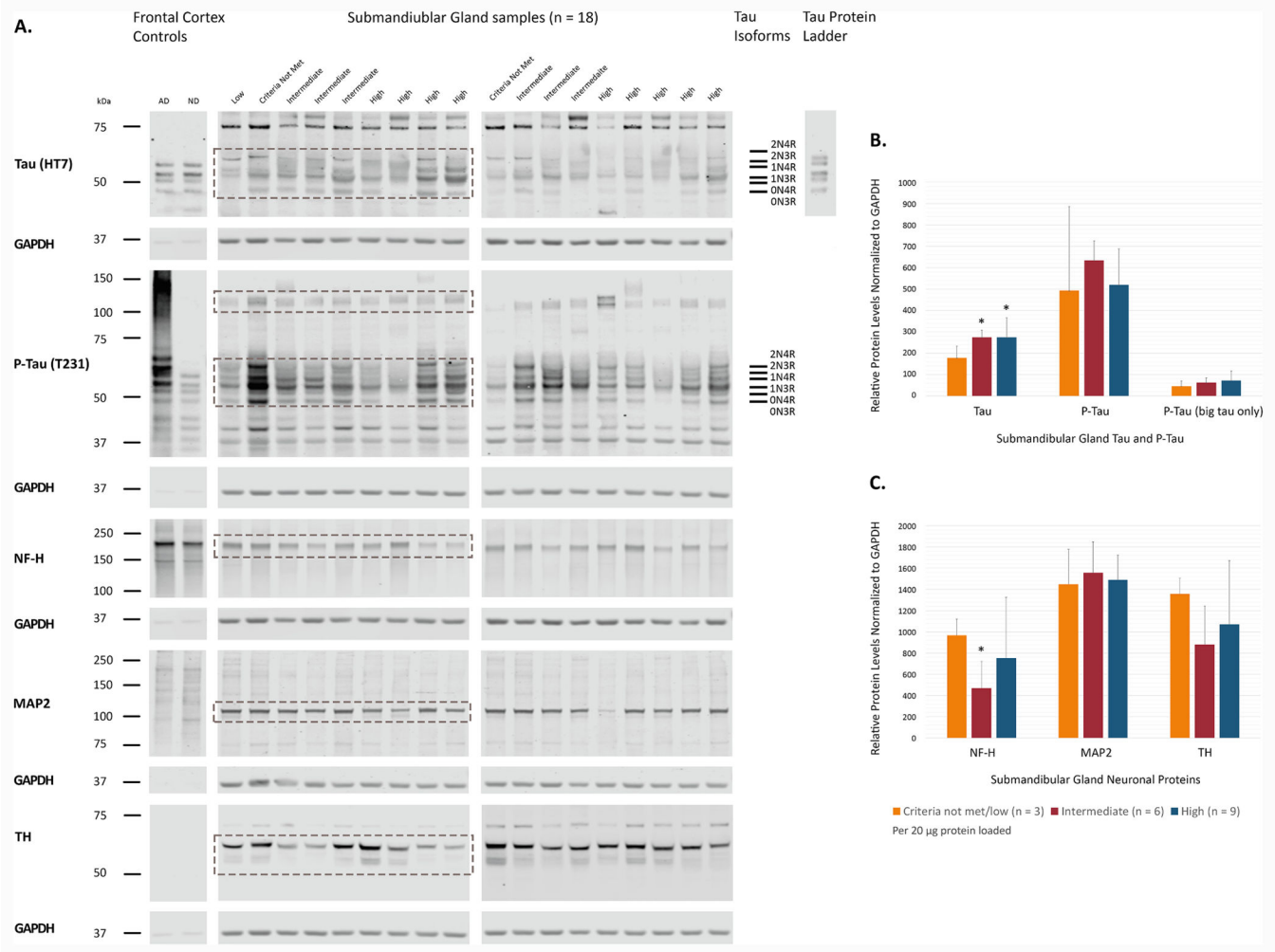

Fig. 1.

Biochemical analysis of the submandibular gland. A. Western blots of Tau, P-Tau, TH, NF-H, and MAP2. Quantified areas are denoted by dashed boxes. On the right, mean and standard deviation (error bars) of Western blot signals with values normalized to GAPDH of B. Tau, P-Tau, and P-Tau (Big Tau measured separately) and C. NF-H, MAP2, and TH. * = Significantly different from criteria not met or low likelihood.

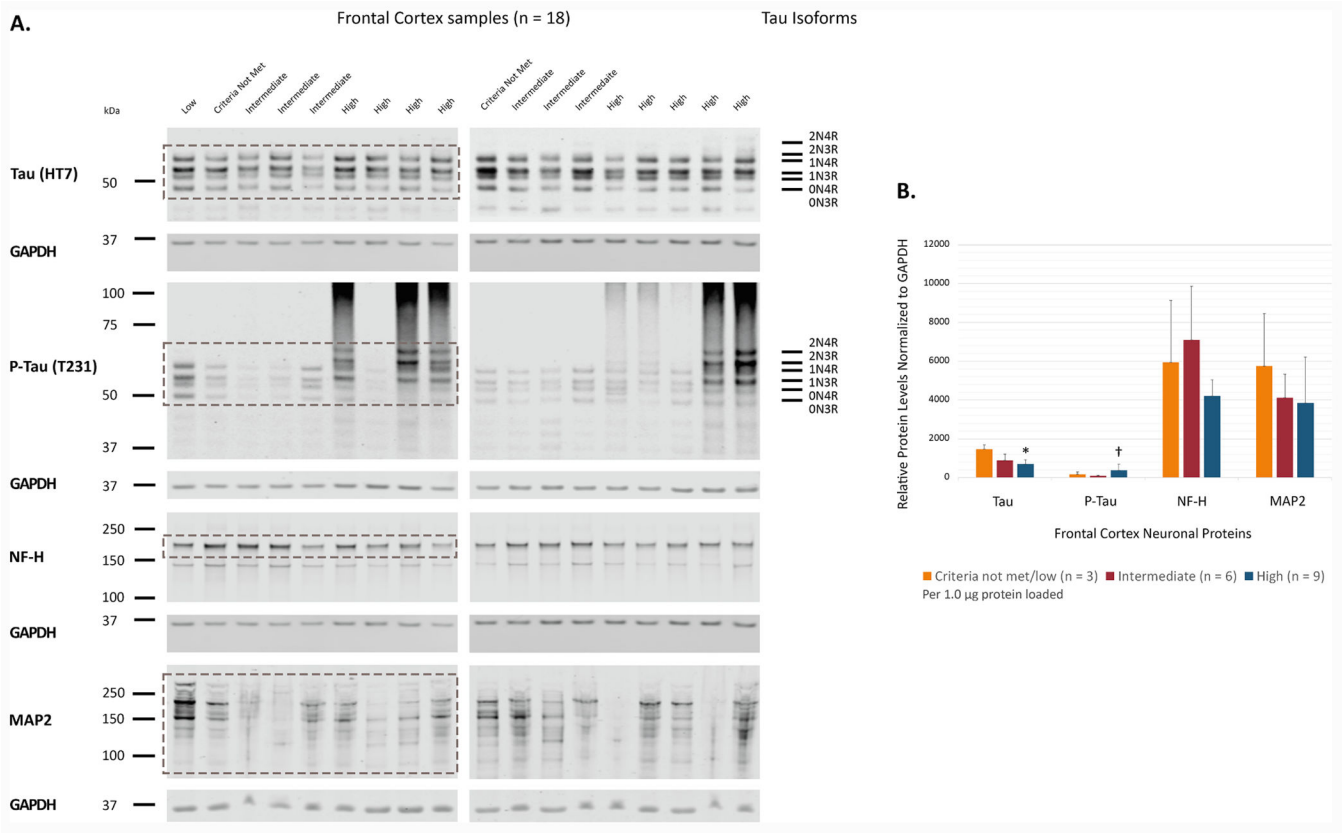

Fig. 2.

Biochemical analyses of the frontal cortex. A. Western blots of tau, P-tau, NF-H, and MAP2. Quantified bands are denoted by dashed boxes. On the right, mean and standard deviation (error bars) of Western blot signals with values normalized to GAPDH of B. tau, P-tau, NF-H, and MAP2, * = Significantly different from criteria not met or low likelihood, † = significantly different from intermediate likelihood.

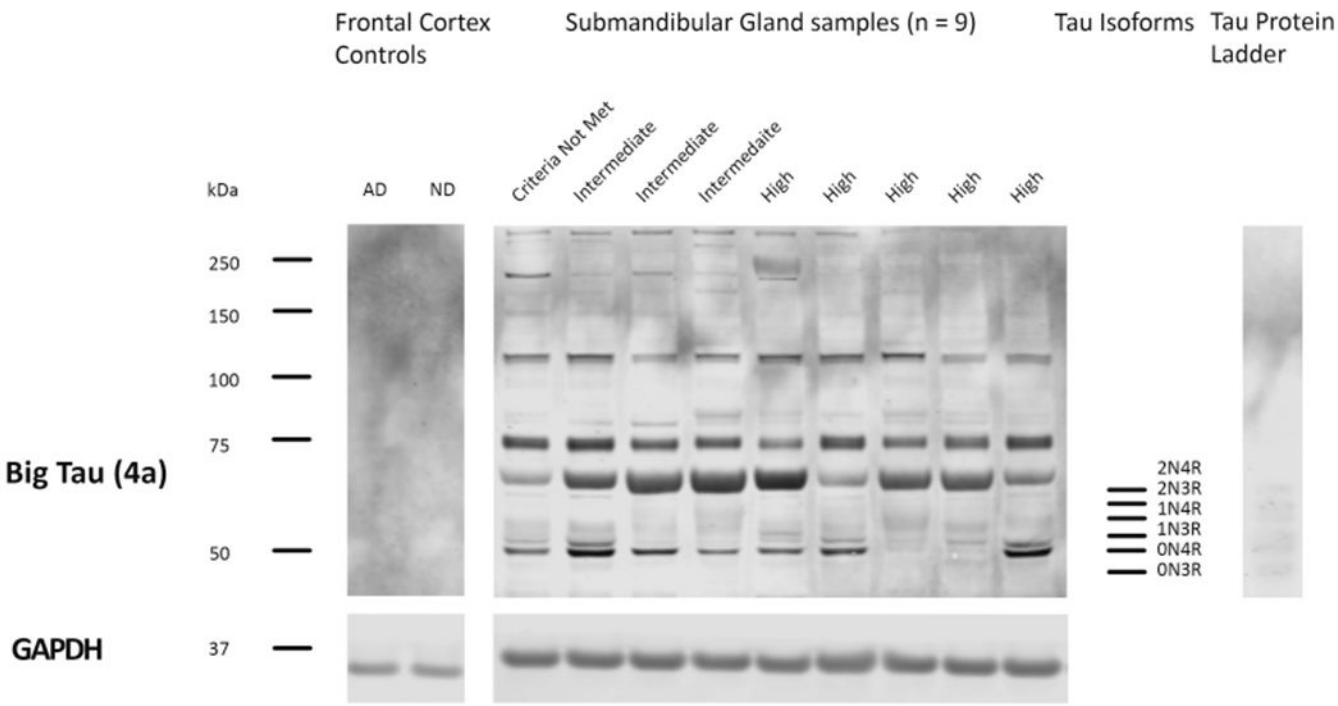

Fig. 3.

Biochemical analyses using a big tau antibody on 9 submandibular gland samples across AD and frontal cortex of an AD and non-demented individual. Denote the high molecular weight species (~110 KDa) present in the submandibular gland samples but not in cortex. Bottom blot is from GAPDH.

Table 1

Subject Demographics. AD = Alzheimer's disease, F = Female, M = Male, NFT = neurofibrillary tangle, PMI = post-mortem interval, Stdv = standard deviation.

Clinicopathological Diagnosis of AD				
	Criteria not met/Low	**Intermediate**	**High**	**Total**
Sample size (n)	3	6	9	18
Average age at death± Stdv, in years	87.3 ± 7.23	89.3 ± 6.44	83.3 ± 4.61	86.0 ± 6.03
# of cases over the age of 90 at death	2	3	1	6
Average PMI± Stdv, hrs	2.7 ± 0.49	3.2 ± 1.01	3.5 ± 0.63	3.2 ± 0.76
M:F	3:0	4:2	4:5	11:7
Braak NFT stage, median (range)	III (II–III)	IV (III–IV)	V (V–VI)	IV, V (II–VI)

Table 2

Primary Antibodies.

Primary Antibodies Target Protein	Notation	Manufacturer	Catalog #	Dilution Used
Tau (HT7)	tau	ThermoFisher	MN1000	1:1000
Phosphorylated Tau (T231)	P-tau	Abcam	ab151559	1:1000
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	ThermoFisher	39-8600	1:5000
Tyrosine Hydroxylase	TH	ThermoFisher	OPA1-04050	1:1000
Neurofilament Heavy Chain	NF-H	Abcam	ab7795	1:1000
Microtubule Associated Protein 2	MAP2	Abcam	ab5392	1:5000
4a exon specific sequence to tau (Mouse)	Big Tau (4a)	Gift from Drs. Itzhak Fischer and Peter Baas	n/a	1:1000

Table 3

Secondary antibodies.

Secondary Antibodies	Antibody Detected	Manufacturer	Catalog #	Dilution Used
IRDye® 800CW Donkey anti-Mouse IgG (H + L)	Tau, NF-H	LI-COR	926-32212	1:10,000
IRDye® 800CW Donkey anti-Rabbit IgG (H + L)	P-Tau, Big Tau (4a), TH	LI-COR	925-32213	1:10,000
IRDye® 680RD Donkey anti-Mouse IgG (H + L)	GAPDH	LI-COR	925-68072	1:10,000
IRDye® 800CW Donkey anti-Chicken IgG (H + L)	MAP2	LI-COR	925-32218	1:10,000

Table 4

T-test for submandibular gland Western blot.

	Criteria not met/low vs. Intermediate	Criteria not met/low vs. High	Intermediate vs. High
Tau	0.004*	0.042*	0.960
P-Tau (50–58 kDa)	0.176	0.577	0.212
P-Tau (110 kDa)	0.249	0.048*	0.941
TH	0.071	0.440	0.505
NF-H	0.017*	0.547	0.282
MAP2	0.632	0.820	0.647

Table 5

T-test alpha values for frontal cortex Western blot.

	Criteria not met/low vs. Intermediate	Criteria not met/low vs. High	Intermediate vs. High
Tau	0.059	0.002*	0.217
P-Tau	0.180	0.244	0.036*
NF-H	0.968	0.143	0.065
MAP2	0.285	0.387	0.861