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Change in Expression of 5-HT₆ Receptor at Different Stages of Alzheimer's Disease: A Postmortem Study with the PET Radiopharmaceutical [¹⁸F]2FNQ1P

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Abstract.

Background: The 5-HT₆ receptor is one of the most recently identified serotonin receptors in the central nervous system. Because of its role in memory and cognitive process, this receptor might be implicated in Alzheimer's disease (AD) and associated disorders.

Objective: The aim of this study was to investigate the binding of [¹⁸F]2FNQ1P, a new specific radiotracer of 5-HT₆ receptors, and to quantify 5-HT₆ receptor density in caudate nucleus in a population of patients with different AD stages.

Methods: Patients were classified according to the "ABC" NIA-AA classification. *In vitro* binding assays were performed in postmortem brain tissue from the healthy control (HC; *n* = 8) and severe AD ("High"; *n* = 8) groups. *In vitro* quantitative autoradiography was performed in human brain tissue (caudate nucleus) from patients with different stages of AD: HC (*n* = 15), "Low" (*n* = 18), "Int" (*n* = 20), and "High" (*n* = 15).

Results: *In vitro* binding assays did not show significant differences for the K_D and B_{max} parameters between "High" and HC groups. *In vitro* quantitative autoradiography showed a significant difference between the "High" and HC groups (*p* = 0.0025). We also showed a progressive diminution in [¹⁸F]2FNQ1P specific binding, which parallels 5-HT₆ receptors expression, according to increasing AD stage. Significant differences were observed between the HC group and all AD stages combined ("Low", "Intermediate", and "High") (*p* = 0.011).

Conclusion: This study confirms the interest of investigating the role of 5-HT₆ receptors in AD and related disorders. [¹⁸F]2FNQ1P demonstrated specific binding to 5-HT₆ receptors.

Keywords: 5-HT₆ receptor, Alzheimer's disease, caudate nucleus, [¹⁸F]2FNQ1P, specific PET radiotracer

INTRODUCTION

Serotonin is reported to have many neurological functions as a neurotransmitter, in the central nervous system but also in many other organ systems:

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cardiovascular, pulmonary, and genitourinary [1]. Serotonin receptor studies identified 7 sub-families of serotonin receptor, with many subtypes in each [2]. The 5-HT₆ receptor is one of the most recently identified, first in rat [3,4] and then in the human brain [5]. The 5-HT receptor family is involved in many physiological processes such as memory, learning, and food intake. Many studies also demonstrated roles in pathophysiological processes, including Alzheimer's disease (AD) [6–15].

The pathophysiology of AD involves several mechanisms, from formation of extracellular amyloid- β plaques [16], to neurofibrillary tangles in the intracellular environment [17], and consecutive neuronal death and synapse loss, all leading to progressive cognitive decline [18]. While the molecule-candidates targeting aggregated proteins have so far been therapeutic failures, a new strategy has emerged in parallel, seeking to target neurotransmission systems more specifically, with the objective of improving the clinical semiology of cognitive decline. An immunohistochemistry study revealed a significant reduction in 5-HT₆ receptor density in cortical areas of AD patients [19], opening up the possibility of specific targeting of this receptor at an early stage of the disease. Several clinical studies evaluated 5-HT₆ receptor antagonists in phase I, II, or III clinical trials for the treatment of AD. Early results were mixed: a phase I study showed the antagonist PRX-07034 to be selective for 5-HT₆, improving short-term memory, while a phase II study reported no benefit of another antagonist (SAM-760) on measures of cognition and neuropsychiatric symptoms in AD patients [20]. Unfortunately, another phase III clinical trial failed, testing idalopirdine, a 5-HT₆ receptor antagonist originally developed for cognitive improvement in AD [21]. However, other 5-HT₆ receptor antagonist compounds are still being investigated [22], such as SUVN-502, currently in phase II trial [23]. Furthermore, 5-HT₆ receptor agonists have paradoxically also been shown to have cognitive enhancement properties [10], leaving this target in the race for symptomatic treatment of AD. Nevertheless, it has to be recognized that research concerning antagonists are more developed than agonists.

In this context, visualizing and quantifying 5-HT₆ receptors during the patient's lifetime through PET imaging would be a valuable contribution to understanding this therapeutic target. The development of a specific radiotracer for 5-HT₆ receptors seems necessary for better understanding of 5-HT₆ receptor

mechanisms in AD, classification of AD grades and long-term follow-up of patients treated with 5-HT₆ antagonists. Several radiotracers have been described in literature, but none stands out as specific for the 5-HT₆ receptor [24, 25]. Our laboratory recently developed a new specific radiotracer for these receptors, [¹⁸F]2FNQ1P. A previous study described the synthesis of its precursor and its radiolabeling [26]. A recent pre-clinical study in non-human primates suggested that [¹⁸F]2FNQ1P is a reliable PET radiotracer for visualization and quantification of 5-HT₆ receptors [27].

Regional distribution and associated neuronal or glial expression of 5-HT₆ receptors were investigated on human postmortem tissue by both autoradiography with [¹²⁵I]SB258585 and immunohistochemistry [28, 29]. These studies revealed high levels in the striatum, moderate levels in the hippocampus and cerebral cortex, and low levels in cerebellum.

The aim of the present study was to investigate postmortem binding of [¹⁸F]2FNQ1P in the caudate nucleus in a population of patients at different AD stages. This region, rarely explored in the context of AD [16], was chosen because of its high density of 5-HT₆ receptors, favorable for quantitative autoradiographic analyses. We hypothesized that 5-HT₆ receptor density would correlate with the stage of AD progression and severity.

MATERIALS AND METHODS

Subjects and tissue samples

Adjacent unstained frozen slices (30 μ m thickness) from human caudate nuclei ($n=68$) were obtained from the London Neurodegenerative Diseases Brain Bank [30]. Sixteen fresh samples of caudate nucleus were also obtained: 10 from the 68 patients of the Medical Research Council (London) Neurodegenerative Diseases Brain Bank and 6 from the Medical Research Council (Lyon) bank (CardioBioTec, Lyon Hospitals) after approval by the hospital department review board. Patient ages, postmortem interval, and sex ratio were not significantly different ($p>0.05$) between patient groups (Table 1). Tissues were collected through appropriate consent procedures under the ethics procedures of the brain banks. Patients were stratified according to the guidelines of the National Institute on Aging-Alzheimer's Association [31] This classification uses an "ABC" staging protocol for the

Table 1
Demographic data of patients

AD Stages	HC	Low	Int	High
Number of cases	15	18	20	15
Age (y)	83.4 ± 4.5	86 ± 4.1	87.3 ± 2.9	83.1 ± 4.2
Gender (M/F)	6/9	7/12	15/5	9/6
Postmortem interval (h)	47.1 ± 13.7	47.8 ± 8.9	51.1 ± 10.4	46.2 ± 13.9

No significant differences were observed between groups for age, sex ratio, and postmortem interval criteria ($p > 0.05$). HC, healthy control.

neuropathologic changes in AD, based on three morphologic characteristics of the disease: amyloid- β plaques (A), neurofibrillary tangles (B), and neuritic plaques (C). Using a system of scores for each group, the ABC score was transformed into four levels of AD neuropathologic alteration: HC (healthy control group), “Low”, “Int” (intermediate), or “High”.

For unstained frozen slices, 15 patients were in the “High” stage of AD, 20 “Int”, 18 “Low”, and 15 HC. For fresh caudate tissues, 8 patients were HC and 8 “High”. The tissues were stored at -80°C and defrosted extemporaneously.

Radiosynthesis of the 5-HT₆ receptor radiopharmaceutical and quality controls

The chemical nitro-precursor of our 5-HT₆ PET radiotracer, [^{18}F]2FNQ1P, was synthesized as described previously [26]. Radiolabeling with ^{18}F was performed extemporaneously, on the days of the *in vitro* experiments, according to our published protocol [32]. Briefly, radiosynthesis used an automated Neptis fluorination module (OOC, Belgium). [^{18}F]2FNQ1P quality control determined radiochemical purity and molar activity on analytical HPLC assay at the end of each production run, guaranteeing the radiopharmaceutical quality of the radiotracer used for the following *in vitro* experiments: i.e., molar activity $> 340 \text{ GBq}/\mu\text{mol}$ and radiochemical purity $> 98\%$.

In vitro binding assays

In vitro binding assays were performed in post-mortem fresh caudate nucleus samples from the HC and “High” groups. Briefly, tissues were preserved in phosphate buffered saline (PBS)/EDTA 0.1% and ground with buffer (50 mM Tris-HCl pH 7.4 at 25°C) (BetaPrion, BSE Purification kit, HMOGEN TUB, biochemistry). Homogenates were centrifuged for 20 min at 35,000 g (Discovery M150 SE ultra-

centrifuge, Hitachi). The pellet was resuspended in 50 mM Tris-HCl (pH 7.4 at 25°C) and incubated for 15 min at 37°C . Following two further centrifugation steps (as above), the membranes were finally resuspended and stored at -80°C until use.

Brain tissues were preserved in buffer containing 50 mM Tris-HCl, 10 μM pargyline, 5 mM MgCl_2 , 5 mM ascorbate, and 0.5 mM EDTA (pH 7.4). Binding assay consisted of 50 μL displacing compound (SB-258585 1 μM : a 5-HT₆ receptor antagonist) or buffer, 100 μL membrane suspension (corresponding to approximately 60 μg protein per well for brain tissue) and 50 μL [^{18}F]2FNQ1P (molar activity, 59.2 GBq/ μmol). [^{18}F]2FNQ1P was used at concentrations from 0.05 to 10 nM. Association rates were determined by incubation of membranes with radioligand at 25°C for 60 min before termination of the experiment. Bound radiolabeled tracer was separated from free tracer by filtration under reduced pressure (MultiScreen HTS-FB, Millipore). Filters were washed 6 times with 200 μL PBS. Washed filters were assayed for radioactivity by γ -counter (Gamma Wizard 2480, Perkin Elmer).

Quantitative in vitro 5-HT₆ receptor autoradiography

In vitro autoradiography was performed on adjacent unstained frozen slices of human brain tissue (caudate nucleus) from patients with different stages of AD. For each patient, pairs of adjacent slices were defrosted. The first slide was incubated for 60 min in buffer containing 50 mM Tris-HCl, 10 μM pargyline, 5 mM MgCl_2 , 5 mM ascorbate, and 0.5 mM EDTA (pH 7.4) and 18.5 kBq/mL [^{18}F]2FNQ1P, to measure total binding, and the second was incubated in the same buffer with SB-258585 at a concentration of 100 nM, for the competition experiment to measure non-specific binding. Each slide contained two adjacent of caudate nucleus sections constituting duplicate for total and specific binding. After incubation, the slices were washed in distilled water,

then dried and placed on a phosphor imaging plate (BAS-5000, Fujifilm) for 60 min. The films were analyzed by a computer-assisted image analysis system (Multigauge, Fujifilm). For each patient, a region-of-interest (ROI) was manually drawn on one of the two sections of the slide to measure the total binding. This ROI was replicated on the other three sections (total binding and non-specific binding) to compare regions with a same size. These ROIs were positioned in homogeneous areas, avoiding folds or tissue damage due to poor sample quality, if any. The mean of total binding values and non-specific binding values (in PSM/mm²) was calculated for each slide. Specific binding was determined by subtracting non-specific binding from total binding values.

In parallel, calibration standards were prepared from rat brain tissue homogenates, as described in a previous study [33]. Briefly, crushed rat brains were homogenized, and proteins were quantified by a chemical analyzer. The rest of the homogenates was frozen at -80°C. For each radiosynthesis, four homogenates were defrosted and mixed with increasing radioligand activities (116, 231, 463, and 925 kBq). The mixture was then refrozen in isopentane

cooled with dry ice. The frozen samples were then cut into 30µm coronal slices using a -20°C cryostat. The slices were placed on the human brain slices on the imaging plates and signal-to-concentration curves were calculated.

Data analysis

ANOVA tests assessed group homogeneity for age, gender, and postmortem caudate tissue sampling interval (Table 1).

In radioligand binding studies, K_D and B_{max} values were calculated using GraphPad Prism software (Graph Pad Software, Prism 6). Statistically significant variations in radioligand binding parameters were assessed on non-parametric Mann-Whitney test. For the quantitative autoradiography study, ANOVA assessed statistical differences. In case of a difference, results were compared between groups with on *post-hoc* Bonferroni correction test. Correlations between AD stages (quantified as: HC = 0; Low = 1; Int = 2; High = 3) and specific binding were assessed on Pearson's r . For all analyses, the significance threshold was set at $p < 0.05$.

Fig. 1. *In vitro* binding assays. Example of saturation binding curves and Scatchard plots with fresh caudate nucleus samples from HC (A) and “High” AD stage (B) using the 5-HT₆ radiopharmaceutical [¹⁸F]2FNQ1P. No significant differences were observed between HC and “High” groups for the dissociation constant (K_D) (C) or number of binding sites (B_{max}) (D; Mann-Whitney test, $p > 0.05$). Bars plot mean and 95% confidence interval.

RESULTS

In vitro binding assays

In vitro binding assay showed reproducible binding of [¹⁸F]2FNQ1P to 5-HT₆ receptors in the HC and “High” groups (Fig. 1A, B). Mean radioligand equilibrium dissociation constants (K_D) were 2.6 ± 2.8 nM and 2.4 ± 1.7 nM the HC group and “High” groups, respectively (Fig. 1C). Total 5-HT₆ receptor density (B_{max}) was 0.19 ± 0.09 and 0.12 ± 0.04 for the HC group and “High” groups, respectively, the difference being non-significant (Fig. 1D).

In vitro quantitative 5-HT₆ receptor autoradiography

Regardless AD stage, all samples showed binding for [¹⁸F]2FNQ1P according to the location of the 5-HT₆ receptor in the caudate nucleus. Figure 2A represents an example of the total and non-specific binding of the [¹⁸F]2FNQ1P depending on the stage of AD. Mean percentage specific binding compared to total binding was respectively 21.1 ± 9.7 , 16.9 ± 15.3 , 16.5 ± 12.6 , and 10.9 ± 15.4 for HC group, “Low”, “Int”, and “High” stages. Results are presented in Fig. 2 according to AD stage. Mean specific binding was respectively 96.1 ± 27.6 , 68.4 ± 41.4 , 70.9 ± 48.7 , and 39.3 ± 43.5 pmol/mg of protein for HC group, “Low”, “Int”, and “High” stages. ANOVA showed a significant difference between groups ($p = 0.004$). *Post-hoc* Bonferroni correction revealed a significant difference between the “High” and HC groups ($p = 0.0025$) (Fig. 2B). *Post-hoc* tests did not reveal significant differences between HC, “Low”, and “Int” groups, but specific binding showed a strong negative correlation with all stages from HC to “High” ($r = -0.42$, $p < 0.01$). A significant difference ($p = 0.011$) was also found between HC group and all AD stages combined (“Low”, “Int”, and “High”) on *post-hoc* *t*-test with Bonferroni correction (Fig. 2C).

DISCUSSION

To our knowledge this is the first study investigating a specific PET radiotracer of 5-HT₆ receptors in a population of AD patients with different stages of disease. The results showed a decrease in caudate 5-HT₆ receptor density with [¹⁸F]2FNQ1P in subjects with AD compared to control group.

Fig. 2. Autoradiographic quantification of specific binding of the 5-HT₆ radiopharmaceutical [¹⁸F]2FNQ1P according to group of patients. A) Example of regional distribution of [¹⁸F]2FNQ1P binding sites in the caudate nucleus in HC and patient groups. The autoradiographs showing the decrease of [¹⁸F]2FNQ1P binding when SB258585 is added during incubation represent non-specific binding. B) A significant difference was seen between the “High” and HC groups (** $p < 0.01$). Bars plot mean and 95% confidence interval. C) A significant difference was also found between HC group and all AD stages combined (“Low”, “Int” and “High”) on *post-hoc* *t*-test with Bonferroni correction (* $p < 0.05$). Error-bars with 95% confidence intervals of the means do not overlap.

As previously outlined, [¹⁸F]2FNQ1P is the only PET radiopharmaceutical currently available that is specific for 5-HT₆ receptors [32]. In addition, [¹⁸F]2FNQ1P has a high specificity for 5-HT₆

receptors with comparison to the following serotonergic receptors, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{1B}, 5-HT₄, as well as for dopamine (D₂, D₃) and muscarinic (α_{1B}) receptors, which are very present in the striatal regions [26]. In this study, binding assays showed reliable affinity of [¹⁸F]2FNQ1P for these receptors. Radioligand saturation binding assays found no significant difference between the HC and “High” groups on the K_D and the B_{max} parameters. The high affinity of [¹⁸F]2FNQ1P for 5-HT₆ was confirmed by the mean K_D for each group (around 2.5 nM). This result is consistent with a previous study which found a K_D of approximately 1 nM [26]. Although B_{max} was lower in the “High” than the HC group, the difference was non-significant. These results could be explained by the small size of the study population (*n* = 8). This explanation is frequently put forward in postmortem studies, as access to brains of neuropathologically documented AD subjects is limited. However, further investigations with a larger sample might demonstrate a significant difference, such as was found in the autoradiography assay.

Quantitative autoradiography experiments showed that [¹⁸F]2FNQ1P was suitable for visualizing and quantifying striatal 5-HT₆ receptor density. *In vitro* competition assays with the 5-HT₆ antagonist SB-258585 showed displacement of the radiotracer, confirming binding reversibility. Quantification of 5-HT₆ receptors revealed significant differences between the healthy control group (i.e., patients without AD neuropathological modifications) and patients with a high level of neuropathological modification (“High”). The results also distinguished the healthy control group from all AD stages. On the other hand, no significant differences were observed between the HC or “High” groups and other stages of AD (“Low” and “Int”). Despite these non-significant differences, the results showed a progressive decrease in the 5-HT₆ receptor expression according to AD stage, with a strong negative correlation between specific binding of [¹⁸F]2FNQ1P and disease stage. Here again, the absence of significant difference between the “High” group and earlier stages of AD can be initially explained by the relatively low number of patients in each group. Larger groups would reinforce the power of the study and could demonstrate significant differences. Another explanation concerns the complexity of assessing AD. Because of the large inter-individual variability found in AD, classification can be difficult. This is why many classifications exist, drawn up by different working groups such as

the IWG-2 classification of the International Working Group for New Research Criteria for the Diagnosis of Alzheimer’s Disease [34] and, more recently, the “ABC” classification of the National Institute of Aging-Alzheimer’s Association (NIA-AA) [31], used in the present study. This classification considers AD as a continuum, needing to be diagnosed in its early stages. However, these criteria remain to be validated. Lowe et al. applied this classification to the Alzheimer Disease Neuroimaging Initiative (ADNI) cohort [35]. The study showed the weakness of the NIA-AA criteria because of the complexity of interpreting biomarkers. Other studies assessing the applicability of this classification in AD patients with mild cognitive impairment showed the same weakness: variability in biomarker interpretation, lack of measurement standardization, and varying results [36, 37]. Furthermore, the “ABC” staging is strongly nested in other diseases contributing to cognitive impairment, such as Lewy body disease, vascular brain injury, or hippocampal sclerosis [38]. Thus, patient groups are difficult to distinguish, making it difficult to show significant differences between neighboring groups.

In addition, “ABC” staging is mostly based on anatomopathological criteria. The classification takes account of onset of A β or amyloid plaques, neurofibrillary tangles, and neuritic plaques, but not of clinical status. However, it is well known that anatomopathological modifications in AD correlate with disease symptomatology. Symptom onset correlates with brain atrophy [39], hypometabolism [40], and neurofibrillary changes [41]. Thus, the correlation between decreased 5-HT₆ receptor density and decrease in symptomatology could be an approach worth considering in future studies.

Another point to be taken into account in interpreting the present results concerns the polymorphism of the 5-HT₆ receptor. The human 5-HT₆ receptor protein is coded by chromosome 1 and the specific gene of the receptor contains many trinucleotide polymorphisms [5]. Some previous studies, in Chinese and Korean populations, showed an association between this polymorphism and AD, and considered this allele to be a risk factor [42, 43]. Another study, in a Caucasian population, found no significant differences between controls and AD patients [44], and considered this 5-HT₆ receptor polymorphism as a silent mutation that does not affect the function of the protein. To date, an association between 5-HT₆ polymorphism in AD patients and the binding properties of [¹⁸F]2FNQ1P is still an open question.

Although the present study is the first to visualize 5-HT₆ receptors in AD patients with a radiopharmaceutical usable for neuroimaging *in vivo*, a few previous studies also showed a decrease in these receptors in AD and related disorders, using dedicated *in vitro* exploration probes. Lorke et al., in an immunohistochemistry study [45], reported decreased cellular expression of 5-HT₆ receptors in the prefrontal cortex of AD patients in comparison with normal age-matched subjects. 5-HT₆ receptors were expressed in pyramidal cells and stellate-shaped cells, and AD patients showed a significant 40% decrease in 5-HT₆ receptor expression. Garcia-Alloza et al. reported similar results. They assessed the involvement of serotonergic disturbance of 5-HT₆ receptors in AD impairment. Binding assays with [¹²⁵I]SB-258585, an *in vitro* radiotracer, were performed on tissue samples from frontal and temporal cortex. Results showed a significant decrease in 5-HT₆ receptor density in both frontal (56% reduction) and temporal (58% reduction) cortex in AD patients compared to controls [19]. While these results were consistent with 5-HT₆ receptor involvement in AD, the authors did not use the clinicopathologic diagnostic classification used in the present study.

The present study also differs from a majority of previous studies in the choice of the brain region of interest: the caudate nucleus, well-known to be a region rich in 5-HT₆ receptors [46, 47]. It is known that 5-HT₆ receptors are highly and homogeneously concentrated in the caudate nucleus, putamen, and nucleus accumbens [29]. The first preclinical explorations of our radiopharmaceutical confirmed its preferential striatal binding [26, 27, 32], justifying the choice of this region for the present postmortem study. However, it must be recognized that the pathophysiological involvement of the caudate nucleus in AD is not yet well established. Brain atrophy and neuron loss occurs mainly in the frontal cortex, hippocampus, and limbic areas [48]. De Jong et al. suggested a distinction in striatal pattern morphology in AD patients compared to subjects with memory complaints without objective cognitive impairment [49]. Their study showed that cognitive impairment is related to the degree of surface deformity, hypothesizing that associative and limbic cerebral networks are primarily affected in AD. These findings highlight the interest of tracing the progression of 5-HT₆ receptors as striatal marker during neurodegeneration.

This is all the more interesting as the 5-HT₆ receptor is also a potential therapeutic target in AD [50].

Recently, drug-candidates have been developed on the pharmacological hypothesis that 5-HT₆ receptor blockade induces acetylcholine release and so improves cognition processes in AD [51]. So far, clinical studies were disappointing. Idalopirdine, a 5-HT₆ receptor antagonist, did not improve cognition in patients with mild to moderate AD compared to placebo [21]; intepirdine, another selective 5-HT₆ receptor antagonist, did not show efficacy in a phase II study [52]. However, these early studies do not signify the abandonment of therapeutic targeting of 5-HT₆ receptors. Their main limitations were the lack of knowledge of 5-HT₆ receptor status in groups of heterogeneous AD patients. The difficulty of staging patients may have led to underdosing in phase III compared to phase II [53]. It is therefore crucial to have better knowledge of the progression of 5-HT₆ receptors during the course of AD, through PET imaging, to highlight potential early biomarkers, to refine the recruitment for future therapeutic trials, and, finally, to contribute to the understanding of 5-HT₆ drug-candidates by drug occupancy studies. The translational results we obtained with the radiopharmaceutical [¹⁸F]2FNQ1P therefore encourage us to implement a PET neuroimaging study in AD patients. Indeed, this *in vivo* exploration will enable us to quantify other brain regions expressing significant densities of 5-HT₆ receptors relevant to AD, such as the entorhinal, parietal, or frontal cortex, and to explore the modification of their expression during the disease progression.

CONCLUSION

This *in vitro* study is the first to demonstrate a decrease in caudate 5-HT₆ receptor density with [¹⁸F]2FNQ1P, a PET radiopharmaceutical, in subjects with AD. The challenge will now be to transfer [¹⁸F]2FNQ1P to *in vivo* PET neuroimaging studies to confirm an early decline in 5-HT₆ receptor expression during disease progression and provide better understanding of the pharmacology of future candidate molecules targeting this serotonergic receptor.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Tissues were collected through appropriate consent procedures under the ethics procedures of the brain banks. Previous consent to do experiments

was given at the time of brain donation, and no supplementary consent was needed for this study. The study was conducted according to the principles of the Helsinki and subsequent revisions.

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Authors’ disclosures available online (<https://www.j-alz.com/manuscript-disclosures/19-1278r2>).

AVAILABILITY OF DATA AND MATERIALS

The data sets supporting the conclusions of this article can be made available upon request through the corresponding author.

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