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Annotation - Does Alzheimer's disease begin in the brainstem?

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Abstract

Although substantial evidence indicates that the progression of pathological changes of the neuronal cytoskeleton is crucial in determining the severity of dementia in Alzheimer's disease (AD), the exact causes and evolution of these changes, the initial site at which they begin, and the neuronal susceptibility levels for their development are poorly understood. The current clinical criteria for diagnosis of AD are focused mostly on cognitive deficits produced by dysfunction of hippocampal and high-order neocortical areas, whereas non-cognitive, behavioural, and psychological symptoms of dementia such as disturbances in mood, emotion, appetite, and wake-sleep cycle, confusion, agitation, and depression, have been less considered. The early occurrence of these symptoms suggests brainstem involvement, and more specifically of the serotonergic nuclei. In spite of the fact that the Braak staging system and NIA-RI criteria do not include their evaluation, several recent reports drew attention to the possibility of selective and early involvement of raphe nuclei, particularly the dorsal raphe nucleus (DRN), in the pathogenesis of AD. Based on these findings of differential susceptibility and anatomical connectivity, a novel pathogenetic scheme of AD progression was proposed. Although the precise mechanisms of neurofibrillary degeneration still await elucidation, we speculated that cumulative oxidative damage may be the main cause of DRN alterations, as the age is the main risk factor for sporadic AD. Within such a framework, β–amyloid production is considered only as one of the factors (although a significant one in familial cases) that promotes molecular series of events underlying AD-related neuropathological changes.

Keywords

aging; Alzheimer's disease; behavioural and psychological symptoms; cerebrospinal fluid; dorsal raphe nucleus; early diagnosis; fetal brain development; neurofibrillary degeneration; serotonin; tau protein

Introduction

Many neurological conditions, and particularly neurodegenerative diseases, involve the selective loss of specific neuronal populations. One notable example is Alzheimer's disease (AD) in which an identifiable subpopulation of large, cortically projecting, pyramidal neurons in high-order regions of the neocortex, are preferentially affected in the course of the disease [1,2]. These neurons share a particular morphologic and neurochemical

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phenotype characterized by their size, distribution, and connectivity and their high expression of dephosphorylated epitopes of neurofilament proteins. They are affected by neurofibrillary tangles (NFTs) formation at very early stages of AD, degenerate at a faster rate than other pyramidal neurons, and shrink significantly in the process [2,3]. However, even more than 100 years after its initial clinicopathological description, no satisfactory explanation for the neuronal specificity of the degenerative process in AD is available.

The amyloid cascade hypothesis began to take shape in late 1980s when a gene on chromosome 21 that produces amyloid precursor protein (APP) was discovered [4]. In short, the *APP* gene contains the sequence for the β–amyloid peptide, which is concentrated in the senile plaques (SPs) used to diagnose AD (Table 1). Some people who inherit a form of early-onset familial AD have a mutation in the *APP* gene that results in the overproduction of the β–amyloid. Similarly, people with Down syndrome, who invariably develop AD in middle age, have an extra copy of chromosome 21 containing the *APP* gene, causing them to produce excess amount of the β–amyloid protein [5]. A variety of enzymes in the brain normally process APP into soluble, seemingly harmless fragments. However, a specific β–secretase cleaves APP in its extracellular domain and leaves a fragment (known as C99) that is secondarily cleaved by the transmembrane γ-secretase complex (that includes presenilin) to cleave APP to yield the β–amyloid peptide. It is now well established that β–amyloid exists as monomers, oligomers, and fibrils. It seems that β–amyloid peptide has a spontaneous tendency to form oligomers that eventually accumulate into diffuse deposits. Diffuse deposits of β–amyloid have been found in large numbers even in subjects whose intellectual status had been evaluated as normal both neuropathologically [6] and by *in vivo* visualization using Pittsburgh compound B (PIB PET, see below) [7]. It is not known how long diffuse deposits may remain asymptomatic in the brains of cognitively intact subjects and why only certain regions of the brain are susceptible to their “maturation” into focal deposits and neuritic (“mature”) plaques (characterized by a corona of degenerating neurites containing tau protein paired helical filaments around an amyloid core). One hypothetical view for maturation of diffuse α–helical deposits of β–amyloid into focal deposits made of firm β–pleated sheet fibrils involves the presence of copper and zinc ions [8]. The presence of unsoluble β–amyloid fibrils in the neuropil is thought to be one of the major mechanisms that compromises axoplasmic flow and induces neurofibrillary changes (see below).

On the other hand, it has been shown that even naturally secreted oligomers of β–amyloid may inhibit hippocampal long-term potentiation *in vivo* [9]. Moreover, it has recently been demonstrated that amyloid oligomers isolated from the brains of AD patients and injected into rodents are capable of disrupting NMDA receptors and impairing memory [10]. Thus, free-floating oligomers may affect synaptic function years if not decades before symptoms of AD appear. On these premises it can be further speculated that soluble amyloid oligomers interfere with neurotrophic factors released into the synaptic cleft, producing anterograde neurodegeneration during the evolution of AD. Obviously, if this concept is correct, the anti-amyloid therapies would be far more effective if started earlier, before the “toxic” oligomers have had time to alter synaptic transmission (at the same time this would confirm that formation of β–amyloid deposits is actually a defense mechanism). However, although most current research still follows the path charted by the concept of amyloid cascade hypothesis, it seems that efforts toward an effective treatment and prevention will require some new perspectives, particularly for sporadic AD. Namely, data collected so far strongly indicate that mutations in *PRESENILIN-1 (PS-1)*, *PRESENILIN-2 (PS-2)* and *AMYLOID PRECURSOR PROTEIN* (*APP*) genes have more widespread effects on broader range of cellular functions compared to sporadic cases [11]. One obvious example is that the enhanced γ-secretase production of β–amyloid$_{1–42}$ is not a feature of sporadic AD.

Current state of knowledge

In recent years it became clearer that cerebrospinal fluid (CSF) biomarkers, particularly phosphorylated tau proteins, demonstrate a higher predictive value over algorithms comprised of clinical, neuropsychological and imaging modalities (MRI, SPECT, PET) [12]. Since the use of some of those biomarkers has been improved to a level that relatively early and reliable diagnosis of AD became possible, CSF examination is becoming a part of the routine diagnostic workup for suspected AD in both clinical and neuropathological evaluation of a patient.

Considering the therapy of AD besides the currently registered cholinomimetic agents for early (*donepezil*, *galantamin*, *rivastigmine*, *tacrine*) and non-competitive antagonist of glutamatergic NMDA receptors (which is also an antagonist of serotonin 5-HT_3 receptors) for moderate/late AD (*memantin*), new drugs, mainly β– and γ–secretase inhibitors, were recently being developed and are currently being tested through various phases of clinical trials in order to prevent the formation and accumulation of the β–amyloid peptide, at least in those subjects with familial early-onset AD (FAD) due to mutations in *PS-1*, *PS-2* and *APP* genes [13]. However, for the vast majority of sporadic, late-onset AD subjects, the neuropathological evidence collected so far does not support the amyloid cascade hypothesis, which is considered too narrow to explain all of the molecular mechanisms that lead to the characteristic accumulation of the neuropathological hallmarks of the disease [14]. It is therefore not suprising that all therapeutic efforts aimed at lowering β–amyloid production and aggregation (including passive and active immunizations) failed to show clinical improvement in AD patients [14].

Unanswered questions

The original amyloid hypothesis contains a conspicious inconsistency that the number of SPs found in elderly brains does not correlate with dementia. On the contrary, substantial evidence has been gathered that the progression of pathological changes of the neuronal cytoskeleton is crucial in determining the severity of dementia in AD [12,15–17]. This fact is strengthened by the numerous reports showing that mutations of *TAU* gene cause hereditary tauopathies with dementia, most notably frontotemporal lobar degeneration (FTLD), in absence of β–amyloid accumulation [18–21]. Although there has been some experimental support for a possible direct causal relationship between the fibrillar β–amyloid [22] or more recently the intraneuronal prefibrillar β–amyloid$_{1–40}$, β—amyloid$_{1–42}$ and β–amyloid oligomers [23–25] and the acute hyperphosphorylation of tau proteins (that is presumed to be continued into full-blown neurofibrillary degeneration) in several cell cultures and transgenic animal models, the exact causes and evolution of human AD-related cytoskeletal changes, their starting point, mechanisms of spreading and the precise causal relationships between plaques and tangles all await elucidation. These problems are also related to the question of AD as a single disease. From the genetic, clinical, and neuropathological point of view, AD is a multifactorial and very heterogeneous disorder [14,26]. Nevertheless, the existence of a common pathophysiological mechanism together with spreading of neurofibrillary degeneration in a more or less orderly fashion in most patients, would justify keeping AD as a single disease as originally believed by Alzheimer himself.

Why only certain types of neurons are preferentially affected in the brain, principally the cerebral cortex, in a layer- and region-specific manner remains unclear. There is ample evidence that in the neocortex, large pyramidal neurons in the deep part of layer III (layer IIIc) and the superficial part of layer V (layer Va), characterized by high somatodendritic levels of nonphosphorylated neurofilament protein are selectively vulnerable in the course of AD [2]. Stereologic analyses showed that their numbers decrease dramatically in cases with

definite dementia compared to other neurons not characterized by this phenotype, to a nearly complete loss (> 90%) in endstage AD [3]. These neurons shrink considerably (by about 50% of their initial perikaryal volume) during NFT formation, the ones larger than 6,000 μm^3 being preferentially affected. Stereologic data show that these neurons are lost at a faster rate in the course of AD than other and smaller pyramidal neurons, which being less prone to NFT formation, may die through other mechanisms in AD, such as oxidative stress, abortive apoptosis (abortosis), and excitotoxic mechanisms [27–30]. Considering that these neurofilament enriched neurons represent only about 30% of the total number of pyramidal neurons in dorsolateral prefrontal cortex [3], their preferential involvement suggests that highly specific cortical pathways are at higher risk for degeneration in AD and that the neurons providing such projections represent important cortical targets for early neuroprotective interventions in AD. In addition to the known alterations of myelin sheath integrity observed during normal brain aging in primates [32], the progressive loss of white matter that is observed over the course of AD [31] ultimately leads to a syndrome of neocortical disconnection. The periventricular white matter changes (leukoaraiosis) that are commonly seen in AD and are associated with low cognitive performance, seem to result from a combination of ischaemia and a failure of CSF to be drained along perivascular pathways that are blocked by the deposition of β–amyloid (cerebral amyloid angiopathy, CAA) and fibrosis in the walls of arteriolosclerotic arteries in the white matter [33]. As a consequence, CSF infuses into periventricular white matter thus increasing signal in T2 weighted MRI images [34,35].

Neuropathological diagnostic criteria for AD

NIA (Khachaturian) criteria

The first neuropathological diagnostic criteria for AD were based on quantification of minimal SPs cortical densities as a function of age [36] (Table 1). They were not broadly accepted because SP formation was later shown to be partly a benign age-related phenomenon (making criteria less sensitive) [15]. Moreover, the cortical regions for quantification as well as the role of the clinical history were not well defined (making criteria less specific and non-comparable) and neurofibrillary changes (NFTs, neuritic plaques, and neuropil threads) were not considered. Therefore, another set of standardized criteria known as CERAD (Consortium to Establish a Registry for Alzheimer's disease) was proposed [37].

CERAD criteria

CERAD semiquantitative criteria were determined as a function of neocortical neuritic plaques (NPs) development in the superior temporal gyrus, prefrontal cortex and lower parietal lobule using modified Bielschowsky or thioflavin S staining in three age groups (less than 50, 50 to 75, and over 75 years) [37] (Table 1). Based on the combination of clinical information and NPs score, three levels of diagnostic certainty were assessed (definite, probable, or possible AD). Besides the fact that the hippocampal formation was again absent from criteria (despite its well known characteristic and crucial involvement in the initial stages of typical AD), the major weakness of CERAD criteria was that they relied chiefly upon the overproduction and accumulation of β–amyloid, and did not consider neocortical NFTs. Unlike SPs, except in low quantities in the entorhinal cortex [38,39], neocortical NFTs are not present in normal aging and were later confirmed (in contrast to SPs) to correlate strongly with dementia severity [12,15]. However, although clinico-pathological correlations showed the best correlation of dementia with the quantity of NFTs, the pattern of synapse and neuron loss in AD does not necessarily match the pattern of NFTs formation [29,38,40].

Braak and Braak staging system

In the meantime, the Braak and Braak Staging System (BBSS) became widely accepted for scoring the neurofibrillary changes in AD [41]. The BBSS classifies the progression of AD neurofibrillary degeneration in six stages, spreading from the transentorhinal region (stage I) to the hippocampal formation (stage II), the temporal, frontal, and parietal neocortex (stages III and IV), and finally to unimodal and primary sensory and motor areas of the neocortex (stages V and VI). Illustration of early neurofibrillary changes in entorhinal and temporal cortex and hippocampal formation in two patients who fulfilled clinical criteria for AD is given in Figure 1. In short, our observations indicate that changes in limbic tau pathology spread in a highly ordered manner from layer II entorhinal neurons to cells located in deeper entorhinal layers, hippocampal formation and temporal isocortex. For example, the epitope Ser199/Ser202/Thr205 (as revealed by monoclonal antibody AT8) has been always found phosphorylated before the Ser396/Ser404 epitope (monoclonal antibody AD2) [42,43], whereas cleaved tau (detected by antibody MN423) could not be found in layers free of phosphorylated tau at these sites [43].

NIA-RI criteria

In an attempt to reconciliate the amyloid hypothesis with the major role of NFT in clinicopathological correlations, in 1997, the more rigorous National Institute on Aging - Reagan Institute (NIA-RI) consensus recommendations for the postmortem diagnosis of AD were issued [44] (Table 1). NIA-RI criteria use CERAD protocols for tissue processing, as well as a semi-quantitative assessment of AD lesions that must be made in several neocortical areas, hippocampus, substantia nigra and locus coeruleus, while also taking into account the BBSS for neurofibrillary changes. In contrast to CERAD criteria, which incorporate clinical data to provide a neuropathological diagnosis, NIA-RI criteria primarily aim to define the probability that clinical dementia was really due to AD-related lesions. Because NIA-RI criteria take into account the apparent number of neocortical NFT, in comparison to CERAD they are more specific but less sensitive, as many AD patients have low numbers of neocortical NFT [45]. As such, definitive neuropathological criteria for AD are still lacking.

The role of reactive processes in the pathogenesis of AD

Generally, the AD pathological changes can be broadly divided into three categories: lesions related to accumulation (“positive lesions”), lesions due to losses (“negative lesions”) and those related to the reactive processes such as inflammation and plasticity (“reactive lesions”) [46]. The losses of synapses and neurons are probably most directly related to the neurological deficits observed in AD patients. However, since they are difficult to estimate and evaluate neuropathologically, so far only the “positive lesions” that are easy to detect constituted the basis for the postmortal diagnotic criteria. On the other hand, the reactive processes are often thought to appear late during the course of the disease and were therefore not considered adeqate to be included in the neuropathological diagnostic criteria for AD.

Neuroinflammation as a possible link between amyloid deposition and neurofibrillary degeneration

It has been recently suggested that microglial activation may occur very early during the course of AD and that neuroinflammation may represent the possible link between amyloid deposition and neurofibrillary degeneration. A recent study investigating plasma biomarkers that could predict conversion from MCI to clinical AD identified raised levels of several cytokines and interleukins associated with inflammation (TNFα, IL-3, IL-1α and IL-11), which are secreted by microglial cells [47]. In turn these pro-inflammatory factors may change the substrate specificity of kinases/phosphatases leading to tau phosphorylation at

pathological sites [48]. Albeit indirect, further evidence to support apossible link between inflammation and AD comes from increased risk of dementia in people who suffered a serious head injury [49,50], patients with cerebral ischaemia [51] and subjects with Type 2 diabetes mellitus and insulin resistance [52,53]. All of these three conditions are associated with increased production of pro-inflammatory molecules and a higher propensity to develop AD or to exacerbate AD symptoms.

The key role of tau protein in development and progression of cytoskeletal neurofibrillary changes and reactivation of fetal plasticity in AD

The key role of microtubule-associated protein tau in AD and related neurodegenerative disorders stems from its roles in neural development and cytoskeleton maintenance. Under normal circumstances, by binding to microtubules, tau regulates their stability and dynamics [54]. The human protein tau is encoded by a single gene located on the long arm of chromosome 17. Tau promoter activity increases significantly during neurite initiation and outgrowth. The human tau primary transcript contains 13 exons of which exons 2, 3 and 10 alternatively spliced and give rise a family of six isoforms [55]. As in aged and cognitively impaired animals the neurofibrillary degeneration due to abnormal phosphorylation of tau protein is scarcely found, the unique expression pattern of tau isoforms in the human central nervous system has been suggested as a possible trade-off between increased cognitive capabilities and decreased resistance to neurodegenerative disorders with tauopathy, especially AD and FTLD [56]. Except for AD and adult cases of Down syndrome that are characterized by both neurofibrillary pathology due to abnormally phosphorylated tau and β–amyloid plaques, all other tauopathies, such as FTLD due to tau mutations, argyrophilic grain disease, corticobasal degeneration and progressive supranuclear palsy (PSP), boxer's dementia, Guam parkinsonism-dementia complex, pallido-ponto-nigral degeneration, and others [57], are characterized by neurofibrillary pathology in the absence of β–amyloid plaques [21,58]. In all of these tauopathies, the tau mutation is sufficiently potent to induce tau aggregation and NFTs in absence of β–amyloid, and is associated with cognitive deterioration and dementia (later appearance of β–amyloid may accelerate this process significantly) [59]. Furthermore, in many sporadic cases of AD, particularly in subjects over 80-year of age, the so-called „tangle only“ AD may be the predominant form [60] comprising about 4% of all AD cases [61].

The molecular species and biological activity of tau is developmentally controlled mainly by phosphorylation of its six isoforms [55,62,63]. The majority of the phosphorylation sites are clustered in the flanking regions of the microtubule-binding domain [55]. In turn, tau proteins change physical characteristics of MTs such as rigidity, length, stability and integrative capacity with other organelles. While normal tau promotes and stabilizes MTs, the non-fibrillized, abnormally hyperphosphorylated tau disrupts MTs, sequesters normal tau, decreases its turnover (due to increased resistance to degradation by calcium-activated neutral proteases, calpains, and ubiquitin-proteosome pathway), misfolds and finally promotes self-assembly into straight or paired helical filaments (PHF), thus impairing axoplasmic flow and leading to slow degeneration [14,64]. In the fetal human brain, there is only one isoform referred to as fetal tau [65], which has three domains (3R) for binding to microtubules (MT). It is thought that the absence of expression of the fourth MT-binding domain during fetal development prevents MTs bundling [14] and allows for the cytoskeletal plasticity required for immature growing neurons [66,67]. Adult tau isoforms have four MT-binding domains (4R tau) and are about 40-fold more efficient at promoting MT assembly [68–70]. The 4R tau protein and particularly the mutated tau protein studied in the most common missense mutations causing frontotemporal dementia with parkinsonism (G272V, P301L, V337M, and R406W) are more easily abnormally phosphorylated and more readily self-aggregate into filaments [67], which may explain an earlier onset and

greater severity of the disease in the inherited FTDP-17 cases. As *in situ* hybridization analysis showed no significant change in the expression of tau mRNA in AD brain [71], the increase in tau level observed in AD brains is probably due to a decrease in its turnover caused by phosphorylation. Out of 80 putative Ser or Thr phosphorylation sites on the longest brain tau isoform, so far 45 of them have been characterized by monoclonal antibodies, mass spectrometry, and sequencing [14,55]. Most of the phosphorylation sites are on Ser-Pro and Thr-Pro motives, but a number of sites on other residues have also been reported [72]. A different states of tau phosphorylation result from the activity of many different specific kinases, of which the most important are probably proline-directed (i.e., acting on serine/threonine followed by proline) kinases glycogen synthase kinase 3β (GSK-3β), mitogen-activated protein kinases ERK 1, ERK2, p70S6 and stress-activated kinases JNK and p38, cyclin-dependent protein kinase-5 (cdk5), and Dyrk1A [14,73] because they have the ability to phosphorylate tau at a large number of sites. Although non-proline-directed protein kinases (such as microtubule affinity-regulating kinase, calcium/calmodulin kinase II, protein kinase A, and others) have been shown to phosphorylate tau at only a very limited number of sites, their importance may be underestimated because phoshorylation of tau by these enzymes significantly increases the phosphorylation of tau by GSK-3β and cdk5 [14]. The decreased activity of at least four different protein phosphatases (PP-1, PP-2A, PP-2B and PP-2C) can also lead to tau hyperphosphorylation in AD [55,74]. Inhibitors of the main phosphatase PP-2A, such as $I_1^{PP\text{-}2A}$ and $I_2^{PP\text{-}2A}$, thus also represent potential therapeutic targets for halting the progression of neurofibrillary degeneration in both AD and normal aging [14].

Hypothesis of reactivation of fetal plasticity in AD

Phosphorylation of tau proteins is high in the fetal period during axonal elongation (Figure 2), and decreases with advancing age due to activation of phosphatases [62,63]. However, after about 30 years of age, yet unknown single or more probably multiple factors such as genetic dysfunction that may include both mutational (*APP*, *PS1* and *PS2*, *TAU* genes) and diverse susceptibility loci (e.g., *APOE*, *α2M*, *αACT*, *LRP1*, *IL1α*, *TNFα*, *ACE*, *BACE*, *BCHE*, *CST3*, *MTHFR*, *GSK3β*, *NOS3*, mitochondrial *CO1* and *CO2*), as well as environmental influences (e.g., head trauma), seem to converge to a single common pathogenic pathway, which cause the phosphorylation of tau to increase again (perhaps together with increased cleavage of tau, which may make tau an even more favorable substrate for abnormal hyperphosphorylation), causing tau polymerization and formation of straight and paired helical filaments that form NFTs, dystrophic neurites and neuropil threads [14]. Impaired brain glucose metabolism has also been shown to facilitate abnormal tau hyperphosphorylation in cultured cells and in mouse models of sporadic AD by at least two different mechanisms, abnormal glycosylation of tau [75] and impaired insulin receptor signalling cascade [76].

The genetic influences on lifespan were shown to be minimal prior to age 60 but increase thereafter [77]. Clearly, the aging process itself contributes to AD. However, AD should not be regarded as “accelerated aging” considering that many people live to extreme age with only minor loss of mental abilities. The autopsy performed on the world's oldest woman, who lived to be 115 years old, reported that her brain was virtually free of neuropathological signs of AD [78]. Besides the cumulative oxidative damage by reactive oxygen species (ROS) [79], which can be provoked by β–amyloid or its fragments [80], some of the hypotheses on putative initial triggers that may activate and speed-up AD-related pathological processes include proposal of neuronal failure due to plasticity burden [81], the potentially detrimental effect of Herpes simplex virus either through the activation of Toll-like receptor pathways in astrocytes and microglia or a change in the phosphorylation state of tau [82–84], brain ischaemia and ischaemic blood-brain barrier [85], and genetically

programmed adaptive metabolic reduction [86]. One of the most plausible concepts that links plaques and tangles in a causative way stems from the idea that the key pathogenetic event and the main mechanism that induces physical damage to axons thus compromising axoplasmic flow is the fibrillogenesis of diffuse β–amyloid deposits (see above) [87]. Neuronal response to this kind of injury results in axonal sprouting [88], upregulation of tau mobilization and its excessive phosphorylation. In order to find the proper path to its targets and form synapses, in these regenerative processes of sprouting and pruning, axonal growth cones and their core MTs need to be less stable [88]. In other words, to induce this level of MTs plasticity, tau proteins need to be in a more phoshorylated state. As we found that the same epitope revealed by the antibody AT8 (Ser202/Ser202/Thr205) is phosphorylated both in growing fetal axons and in degenerating axons of AD brains (compare Fig. 1 and Fig. 2), and taking into account the fact that AT8-immunoreactivity permits evaluation of neuronal changes well before the actual formation of neurofibrillary tangles and neuropil threads [30,89], we speculate neurofibrillary degeneration at least in its earliest stages may include pathological activation of some fetal proline-directed protein kinases, such as one of 100 kDa weight that phosphorylates Ser262 of tau protein [90]. In turn, such kinases may reactivate fetal plasticity mechanisms through phoshorylation of tau protein, this time however in degenerating axons of AD brains, further contributing to the development and progression of AD-related pathological changes.

Clinical criteria for AD and problems of antemortem diagnosis

Clinical criteria for AD and the concept of amnestic mild cognitive impairment (MCI)

The introduction of potential new treatments and drug-modifying therapies such as γ- and β-secretase inhibitors, blockers/inhibitors of β–amyloid aggregation and fibrillization, microtubule stabilizers, muscarinic agonists and others that are supposed to have the best effect if introduced early, as well as potentially better symptom-management therapies (5-HT1a agonists and antagonists, 5-HT4 agonists, mitochondrial permeability modulators, calcium channel blockers), increased awareness about AD in the population and have facilitated patients and their families to seek medical advice earlier during the course of the disease [91]. Therefore, in recent years, the preclinical phase of AD with mild memory impairment, but without overt dementia, has increasingly attracted attention.

According to all clinical criteria recommended so far, AD cannot be diagnosed until dementia is present. Although the NINCDS-ADRDA criteria [92] have a relatively high accuracy rate (around 80–90%) [93,94], these percentages come from specialized expert research academic centers and are based on patients in later stages of the disease who were followed longitudinally for several years before autopsy. In clinical settings, there is no clinical method to determine which patients with mild cognitive impairment (MCI) will progress to dementia, except for a very long clinical follow-up.

Mild cognitive impairment is an aetiologically heterogeneous potential precursor stage of AD, defined by cognitive impairment, that can be shown by objective neuropsychological measures adjusted for age and education. About 40–60% of amnestic MCI patients develop AD during the first 5 years [95], whereas the remainder of MCI patients have a less progressive form of memory impairment [95,96]. It shoud be kept in mind that other types of dementia, such as dementia with Lewy bodies, vascular dementia, and argyrophilic grain disease (Braak's dementia), may also be preceded by MCI [96,97]. The amnestic subtype of MCI (i.e., memory complaint with objective memory impairment, but with preservation of general cognitive functioning without or with minimum impairment of activities of daily living) mostly represents prodromal AD [98], which is confirmed neuropathologically [99].

The major pitfall when studying CSF biomarkers to predict AD in MCI cohorts is the fact that conversion from MCI to AD is only about 12–15% per year (in contrast, only 1–2% of healthy older populations convert to AD per year) [95]. Therefore, only an extensive follow-up time (>5 years) of patients with stable MCI might further increase the specificity of CSF biomarkers [12]. On the other hand, neither the clinical nor the pathological aspects of AD evolve in a linear manner, but the predictable sequence of AD pathology allows for stage-based correlations with cognitive and behavioral symptomatology [100]. Besides CSF biomarkers, future neuropathological criteria and recommendations should therefore include more sensitive and specific immunohistochemical and immunocytochemical subgrouping [101] and neuropathological grading of AD.

Another crucial problem of antemortem diagnosis is that the reliability of both clinical and neuroimaging methods is a function of disease severity, and therefore, there is a risk of increasing overlap with non-AD pathology, psychiatric illnesses, and healthy aging (ICD-10, DSM-IV-TR, NINCDS-ADRDA) [92,102,103], particularly in presence of comorbidities and cases of atypical AD syndromes, such as anterior (frontotemporal) and posterior (parietotemporo-occipital) variants of AD, focal, lobar or gyral progressive degenerative syndromes [104]. Moreover, the clinical spectrum of symptoms of these conditions more closely corresponds to the functional anatomy of the affected brain areas than to the underlying pathology.

The current clinical criteria for diagnosis of dementia in AD are focused mostly on cognitive deficits produced by dysfunction of hippocampal and high-order neocortical areas [92,102,103]. In other words, during the neuropsychological part of the diagnostic procedure, clinicians are looking for impairment of memory for recent events (Braak stages I and II), impaired recall, delayed word recall and word finding difficulties, disorientation in time and space, and impaired concentration and comprehension (Braak stages III and IV), and disturbances in perceptual and motor skills (Braak stages V and VI) [41]. These neuropsychological findings are scored as number of points that, in the most commonly used Mini-Mental State Examination (MMSE), ranges from 0–30 points, where 30 reflects normal mental status across many cognitive domains [105]. Although many other tests for neuropsychological screening for AD are available, they are not used as much as MMSE, which has been criticized along several lines, including sensitivity to educational level, poor sensitivity and specificity, and poor negative predictive value (NPV) and positive predictive value (PPV), especially in early-stage disease [106] (see below).

Imaging modalities to diagnose MCI or early dementia in AD are tightly bound to the measurement of the structural and functional changes in the cerebral cortex, but not other, subcortical structures (see below why authors think this should be changed). Annual rates of cortical atrophy were found to be significantly different in normal aging (0.2–0.5%) and AD (2–3%) [107], particularly in the entorhinal cortex and hippocampus, as revealed by structural MRI volumetric measurement [108–112]. An even earlier characteristic sign of incipient AD is temporoparietal hypometabolism, as assessed by [^{18}F]fluorodeoxyglucose (FDG) PET [113–115]. Recent neuroradiological advances now also allow the measurement of alterations in the white matter integrity in patients with MCI and AD using diffusion tensor imaging (DTI) [116], diffusion tensor tractography (DTT) [117] and the *in vivo* investigation of amyloid pathology, visualized by Pittsburgh Compound B PET (PIB PET) [118,119], while neurofibrillary tangles and neuritic plaques may be visualized using 2–(1–(6–[(2–[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl)ethylidene)malononitrile ([^{18}F]FDDNP PET) [120–123]. Comparisons of PIB PET and FDG PET in AD have shown a reciprocal relationship most clearly in parietal regions, while studies in MCI subjects have shown a bimodal distribution of increased PIB binding, with one group demonstrating PIB retention at the level of AD subjects and the other only non-specific binding [124,125]. Although

differences in binding patterns between patients with AD, MCI and controls were reported to be more pronounced for ^{11}C-PIB than for ^{18}F-FDG PET [126] and for ^{18}F-FDDNP [125], a positive relationship between the severity of depression and anxiety symptoms and ^{18}F-FDDNP binding values that was found in nondemented middle age and older individuals, suggested a potentially important relationship between relatively mild mood symptoms and biomarkers of cerebral amyloid and tau deposition [123].

Clinicopathological correlations of behavioural symptoms and pathology of raphe nuclei

Behavioral and psychological symptoms in AD

Interestingly, in a study of 1,070 non-demented individuals, those who had depressed mood at baseline were found to have an increased relative risk of dementia of 2.94, even after adjustments made for age, gender, educational level and language [127], while in a longitudinal study of 235 patients with early probable AD only 8.5% were free of non-cognitive, so-called Behavioral and Psychological Symptoms of Dementia (BPSD), such as disturbances in mood, emotion, appetite and wake-sleep cycle, “sundowning”, confusion, agitation, depression and others, during the first 3 years of follow up [128]. A retrospective review of 100 autopsy-confirmed AD cases found that, on average, depression, mood change, social withdrawal and other BPSD were documented more than 2 years before the diagnosis of AD was made (the earliest non-cognitive symptom appeared, on average, 33 months before diagnosis) [129]. The early occurrence of BPSD is suggestive of early brainstem involvement and more specifically of serotonergic nuclei. However, although many published investigations initially reported early involvement of the brainstem nuclei by neurofibrillary degeneration during the course of AD (particularly the raphe nuclei - see Table 2), due to the fact that BBSS did not include raphe nuclei in postmortem investigation of AD-related pathology (BBSS criteria was later incorporated into NIA-RI criteria for neuropathological diagnosis of AD), their pathology in AD has not been systematically studied. Because age is the main risk factor for sporadic AD, for us it is logical to assume that a cumulative oxidative damage by ROS, particularly of mitochondrial DNA [130] and RNA (RNA seems to be more susceptible to oxidative insults than DNA since RNA is largely single-stranded, its bases are not protected by hydrogen bonding and specific proteins and it locates in the vicinity of mitochondria, the primary source of ROS) [131], together with other susceptibilities for “wear and tear” of somatic cells, may represent the main potential cause of neurofibrillary degeneration of neurons in DRN. Such a situation resembles the centrality and early involvement of substantia nigra in Parkinson's disease. Within such a framework, β–amyloid should be considered just as one of the factors (although obviously a very significant one in familial cases) that promote or speed-up the molecular series of events underlying AD-related neuropathological changes.

The possible role and the significance of the early dorsal raphe nucleus involvement during AD

The human raphe nuclei consist of the rostral subdivision that gives rise to the ascending projections and caudal subdivision (nuclei raphe magnus, pallidus, and obscurus) that projects into the brainstem and spinal cord. The rostral subdivision comprises the centromedian part (including the annular, linear, and central raphe nuclei) and the dorsal part. The dorsal part, also known as the dorsal raphe nucleus (DRN) contains the largest aggregates of serotonergic neurons (about 80%) and is divided into the interfascicular, supratrochlear, and caudal subnuclei [132,133].

Before the introduction of BBSS many investigators reported AD-related pathology and cell loss in the raphe brain stem nuclei (in addition to those in the basal nucleus of Meynert and

entorhinal cortex) during the early course of AD [134–145] (see Table 2). However, most of them used biased methodology of estimation of nerve cell loss and frequency of tangles from densities assessed, while sampling was done usually only at the level of trochlear nucleus, thus preventing firm conclusions to be made (see Table 2). In spite of the fact that after the introduction of BBSS the raphe brainstem nuclei were not in the focus of investigation, several reports, including some of which used unbiased methodology for quantification, again drew attention to the possibility of their selective and early involvement, particularly the dorsal raphe nucleus, in the pathogenesis of AD [146–159] (see Table 2).

Braak and colleagues later confirmed very early AD-related cytoskeletal changes in the DRN in 27 AD cases [160] (see Table 2). They claimed that in accordance with the cross-sectional data available to them, the ascending system source, the rostral raphe group, is affected as early as in stage I – some 30 years prior to dysmnesia – by the AD-related cytoskeletal lesions [160]. Furthermore, it was recently reported that the DRN may actually display the earliest AD-related cytoskeletal pathology [159] (see Table 2). In this clinicopathological series of 118 cases, out of which 38 were categorized as Braak stage 0 (at least four sections at different levels of transentorhinal cortex were free of neurofibrillary changes, based on immunonegativity for monoclonal antibodies PHF-1 and AT8), and 80 as Braak stage ≥ 1 (rare neurofibrillary changes in the transentorhinal cortex are considered as Braak stage 1), more than 20% of Braak stage 0 and all of Braak stage ≥ 1 cases had substantial neurofibrillary changes in the DRN. An illustration of early neurofibrillary changes of the DRN is given in Fig. 3. Therefore, the putative staging scheme updated with the DRN (and considering the DRN as the first brain structure affected by neurofibrillary pathology and the “earliest source” of transneuronal degeneration), the crude scheme of differential susceptibility levels for neurofibrillary degeneration should be as follows: **dorsal raphe nucleus** --> **entorhinal cortex** --> **hippocampal formation** --> **amygdala** --> **orbitofrontal and prefrontal cortex** --> **posterior association cortical regions** --> **primary sensory and motor cortical areas** (from highest to lowest susceptibility). However, because the raphe nuclei dysfunction due to neurofibrillary changes is not included in the BBSS, its possible behavioural correlates are not yet considered as a potential early characteristic clinical feature of AD. As a matter of fact, studies of the brainstem in AD generally do not pay much attention to the connectivity of the affected nuclei and related functional consequences. In a similar fashion, the molecular characteristics of the dorsal raphe alterations are not yet determined (see below), and cannot be used for diagnostic purposes.

Summary and future prospects

In summary, research studies conducted so far indicate that AD in familial cases due to known mutations in *APP*, *PS1* and *PS2* genes is probably caused by a pathological cascade of events in which formation of β–amyloid oligomers lies upstream of neurofibrillary degeneration. Neuropathologically, some of the familiar AD forms are indeed of the “plaque only type”. Since the “toxic” β–amyloid oligomers are first being accumulated as diffuse deposits in the neuropil and later as SPs, this AD pathology is, however, an end-stage effect rather than cause or at least partially a response to initial insults, thus representing an adaptive, protective reaction [161,162]. Despite intensive efforts to find the mechanistic missing link between plaques and tangles, the steps that connect β–amyloid and its potentially toxic oligomers to cytoskeletal tau pathology still remained largely undefined. On the other hand, in many or even most sporadic cases neurofibrillary degeneration can be caused in the absence of amyloid pathology. Thus a more coherent scheme in these cases can be based on the centrality of the cytoskeletal abnormalities [18,163]. According to this view, the hyperphosphorylation of tau protein aids in both the development and progression

of AD, while regional differences in compartmentalization and metabolism of tau protein possibly represent a molecular basis for individually and regionally different neuronal vulnerability in AD [164]. As it has been documented that hyperphosphorylated tau, but not PHFs, inhibits regeneration of MT network in cultured cells, the formation of PHF may be considered as a self-defense reaction (similarly to the formation of SPs) by the affected neurons [165]. The relationship between the quantity and distribution of NFTs, the loss of neurons and dementia, is not a linear one [29]. This could partially be attributed to the fact that due to the redundancy of nervous elements and compensatory structural, physiological and biochemical processes, a loss of neurons is to a certain limit tolerated without any functional consequences [18]. An important source of discrepancy among studies arises from the fact that most AD studies have focused on elderly people without considering the possibility of a gradual neuronal loss over adult life prior to the occurrence of AD-related changes. As long as elderly patients do not suffer from AD, they appear neuropathologically quite comparable as a group [38,166]. It is therefore not surprising that significant neuron loss due solely to aging cannot be revealed without younger adult cases included in the regressions [167]. On the other hand, when neuronal loss attributable to aging is superimposed to an unbiased estimate of the number of NFTs in AD, regions like the entorhinal cortex and hippocampal formation may display neuronal losses larger than that accounted for by NFT counts alone [18,29,167–169]. It can be assumed that the pattern of neuron loss does not necessarily match the pattern of NFT formation due to mechanisms other than neurofibrillary degeneration [18,29,38,39].

Based on CSF levels of proteins associated with SPs and NFTs, i.e. β–amyloid, total tau and ubiquitin, five different subgroups of AD have already been proposed [170]. As there is a tendency for phosphorylated tau proteins to behave differently in the different primary dementing disorders, the phospho-tau proteins in CSF come closest to fulfilling criteria of biological markers of AD [12]. It is therefore likely that additional subgroups of AD may be identified from phosphorylation patterns of CSF tau. This would help preclinical identification of specific signaling pathways involved selectively in different subgroups of AD patients and also facilitate the development of more specific therapeutic drugs.

In the context of early involvement of the raphe nuclei in AD degenerative process it would for instance be relevant to assess whether 5-HIAA (5-hydroxyindoleacetic acid), the main metabolite of serotonin in CSF, is decreased in patients with BPSD typical for very early AD and whether therefore it could be used as an early biological marker. Perhaps of equal importance would be to test for the functional correlation of genes related to serotonergic transmission that might be altered during the early course of AD by genome and transcriptome microarray analysis of blood and postmortem brain samples. A compensatory mechanism illustrated by an up-regulation of serotonergic metabolism has been shown at the stage of amnestic MCI in contrast with a dramatic decrease at later stages of AD [171]. This difference of hippocampal serotonergic receptor labeling allows distinguishing of patients with amnestic MCI from those with mild AD. Exploring 5-HT1a receptors with 2'-methoxyphenyl-(N-2'-pyridinyl)-p-(^{18})F-fluoro-benzamidoethylpiperazine PET may therefore be useful for better understanding of pathophysiologic changes at early stages of AD [171].

The serotonergic neurons in the DRN can also be easily visualized and quantified by immunohistochemistry using monoclonal antibody PH8 raised against phenylalanine hydroxylase, which cross-reacts with tryptophan hydroxylase, the serotonin synthesis enzyme [148]. Expanding our understanding of the raphe nuclei, particularly the DRN involvement in the early stages of AD to functional concepts beyond neuropathological descriptions will likely have a strong impact on our understanding, detection and tracking of AD progression, and on the development of new therapeutic strategies.

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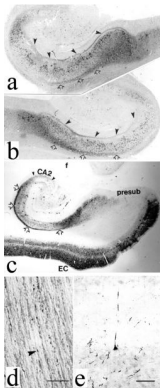

Fig. 1.

Illustration of neurofibrillary changes, which in AD spread in an anterograde fashion through identified neuronal circuits due to yet unknown cause(s). All figures were obtained using the mouse monoclonal antibody AT8 (Innogenetics, Temse, Belgium) used at a 1:200 dilution in indirect immunocytochemistry. It should be stressed that AT8 reacts with tau only when multiple sites around Ser202, including Ser199, Ser202, and Thr205, are phosphorylated. Single phosphorylation of any of these residues is not enough for AT8 reactivity. Thus, AT8 immunoreactivity is useful in detecting phosphorylation of Ser202/Ser202/Thr205 for proline-directed kinases. **a**. Left hippocampus of 73-year old AD man who fulfilled NINCDS-ADRDA and DSM-IV-TR clinical criteria for AD and had a 7-year history of slowly progressive cognitive deterioration and various behavioral symptoms (middle level of hippocampal body, cause of death was bronchopneumonia), black arrowheads show perforant pathway fibers (originating from layer II islands of entorhinal cortex) that contact the apical dendrites of CA1 pyramidal neurons directly, open arrows denote subicular axons, magnification 18.75x; **b**. Right hippocampus of 83-year old woman with AD (middle level of hippocampal body), who had behavioral and memory problems for 5.5 years before death from myocardial infarction and was also fulfilling NINCDS-ADRDA and DSM-IV-TR clinical criteria for AD; **c**. Left hippocampus of the same case as in **a**; black arrowheads delineate CA2 region (most tangle-bearing neurons are not AT8-immunoreactive). Legend: prosub, prosubiculum; EC, entorhinal cortex; magnification 10x. **d**. Beaded-like appearance of AT8-positive axons, same subject as in **a** and **c**, scale bar = 10 μm, **e**. Early neurofibrillary changes of a temporal neocortex layer V pyramidal neuron, scale bar = 100 μm.

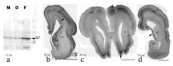

Fig. 2.

Tau protein phosphorylation during fetal brain development. **a**. Western blot of tau protein at 11weeks of gestation (w.g.) using monoclonal antibody AT8. Arrow shows positive bands from all three regions examined, particularly the frontal lobe, **b**. Arrow points to prominent AT8-immunoreactivity in the lower subplate zone of the frontal regions of the telencephalon at 18 w.g., **c**. At 20 w.g. AT8-immunoreactivity moves from lower to the upper subplate zone, **d**. During mid-gestation, the fornix as well as subset of callosal commissural fibers are unambiguously AT8-immunoreactive, whereas the hippocampal formation and internal capsule remained unstained; AT8-immunoreactivity then gradually appears in the cortical plate, diminishing and disappearing completely from the subplate zone at the end of 32 w.g. Fetal brains were part of the Zagreb Neuroembryological collection. Legend: M, mesencephalon, O, occipital lobe, F, frontal lobe, CP, cortical plate, SP, subplate, CI, internal capsule, GE, ganglionic eminence, CC, corpus callosum, Fx, fornix, HF, hippocampal formation, scale bars in b, c and d = 1 cm.

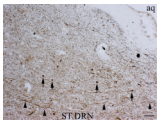

Fig. 3.

Gallyas silver iodide staining of the dorsal part of the supratrochlear subnucleus of the dorsal raphe nucleus of a mildly cognitively impaired 69-year old woman. This method specifically stains paired helical filaments (PHFs) in neurofibrillary tangles (NFTs) (arrows) and degenerated neurites (arrowheads). An initial degree of Gallyas-positive cytoskeletal changes in the dorsal part of ST DRN is seen. Legend: ST DRN, supratrochlear subnucleus of the dorsal raphe nucleus; aq, cerebral aqueduct (of Sylvius). Scale bar = 100 μm.

Table 1

Chronological list of major neuropathological criteria and recommendations for neuropathological diagnosis of AD.

Author(s) and year	Criterion name and its main feature	Major advances	Major drawbacks	Conclusion
Khachaturian et al., 1985	NIA criteria: quantification of SPs cortical densities	First widely accepted criterion	Clinical history and NFTs were not considered	Unsatisfying because SPs were shown to be partly a benign age-related phenomenon (Arriagada et al., 1992) and since clinical history, NPs and NFTs were not taken into account
Mirra et al., 1991	CERAD criteria: semiquantitative assessment of the highest density of NPs in superior temporal gyrus, frontal cortex and parietal lobule from 0 (none) to C (abundant)	Combinationa of clinical history and NPs score; four levels of diagnostic certainty: normal brain, possible AD, probable AD and definite AD	NFTs and hippocampal formation are not considered	Criteria were abandoned because NFTs were shown to correlate best with dementia severity (Arriagada et al., 1992; Bierer et al., 1995; Grossi et al., 2007)
NIA-RI, 1997	NIA-RI criteria: integrate CERAD and Braak criteria (Braak and Braak, 1991) thus evaluating „likelihood“ of AD-changes to cause dementia	Three levels of probability were proposed: high (CERAD NP C – abundant / BBV or VI), intermediate (CERAD NP B – moderate / BB III or IV) and low (CERAD NP A – sparse / BB I or II); include semiquantitative assessment of AD lesions in hippocampus, nucleus niger and LC	There is a considerable number of demented patients with AD who have low numbers of neocortical NFTs	The combination of BB NFTs stage equal to or above IV and CERAD NP stage C together with CDR equal to or above 1, provides the highest sensitivity and specificity; NIA-RI criteria are more specific than CERAD, but less sensitive

Abbreviations: AD, Alzheimer's disease; BB, Braak and Braak stage; CDR, Clinical Dementia Rating; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; LC, locus coeruleus; NIA, National Institutes of Aging; NIA-RI, National Institutes of Aging – Reagan Institute; NFTs, neurofibrillary tangles; NPs, neuritic plaques; SPs, senile plaques

Table 2

Chronological list of selected articles relevant for the transneuronal spreading of AD-related cytoskeletal changes

Author(s) and year	Goal / Hypothesis	No. AD	No. CON	Major findings / Premises	Conclusion
Mann et al., 1983	The goal of the study was to determine the role of serotonin-producing cells in AD by counting neuron numbers of the DRN	20	22	The major finding was 12% cell loss in DRN in AD	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed, together with sampling done only at the level of trochlear nucleus prevents drawing firm conclusions
Curcio and Kemper, 1984	The goal of the study was to determine neurofibrillary changes and neuronal packing density in DRN	7	6	Although cell density was similar between the groups studied, authors determined 8% loss of large neurons in the AD group	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed, together with sampling done only at the level of trochlear nucleus prevents drawing firm conclusions
Yamamoto and Hirano, 1985	The goal was to determine the number of NFTs and neurons in the DRN in AD	5	7	Authors reported 72% decrease in DRN cell density and 77% loss of large neurons in AD patients as compared with controls	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed, together with sampling done only at the level of trochlear nucleus prevents drawing firm conclusions
Pearson et al., 1985	A hypothesis was proposed that the distribution of neurofibrillary degeneration in AD may be accounted for on the basis of the connectivity of the involved structures	>14	0	The largest number of NFTs in AD was found to occur typically in the CA1 subfield and subiculum in the hippocampal formation and in the regions of amygdala that have the most extensive interconnections with the entorhinal cortex layers II and IV in the parahippocampal gyrus	Since the entorhinal cortex is heavily interconnected with the layer II-III and V neurons of the temporal and posterior parietal higher order association areas that suffer the most profound degenerative change in AD, the authors concluded that the remarkable specificity of the distribution of NFTs reflects somehow the anatomical connectivity of the structures involved
German et al., 1987	The goal was to analyze immunohystochemically the regional distribution of subcortical nuclei containing NFTs in brains from AD patients	7	3	The tangles were found not only in DRN and LC, but also in many other subcortical nuclei, such as nucleus paranigralis, peripeduncular nucleus, medial parabrachial nucleus and several midline thalamic nuclei	Authors concluded that AD is a disease of the cerebral cortex and the numerous subcortical nuclei which diffusely innervate it; they hypothesized that subcortical nuclei are secondarily affected due to retrograde transport of a cortical pathogen or failure of normal transport of a trophic agent
Hardy et al., 1987	A hypothesis was proposed that the amyloid deposition in NPs and in cerebral blood vessels in AD is caused by neurofibrillary degeneration of central cerebrovascular regulatory neurons in the brainstem and basal forebrain			Two major premises were that: 1) NPs are similarly distributed in the forebrain and 2) NPs are seen in areas (such as striatum and thalamus) that receive axonal projections from the affected cortical neurons	The conclusion was that the brainstem and basal forebrain neurons that control the blood-brain barrier may die as a result of retrogradely transporting pathogenic agent from their axon collaterals involved in NPs formation in the cerebral cortex
Jellinger, 1987	The goal of the study was to assess the number of cells in DRN of early and late AD patients and compare them with controls	22 (13 early AD, 9 late AD)	25	The author reported DRN cell loss of 41% in early and 36.8%-55.8% in cases of late AD	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed prevents drawing firm conclusions
Saper et al., 1987	A hypothesis was given that a degenerative process in AD spreads transneuronally from specific subcortical			Premise: several subcortical cell groups were found to contain large number of NFTs in AD and all have extensive	Authors hypothesiued that AD is a result of transneuronal degeneration and speculated that AD may somehow involve either the

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	cell groups (LC, DRN, A10 dopaminergic cell group, lateral hypothalamic area and n. basalis) to limbic and paralimbic cortex			cortical projections, particularly to the limbic and paralimbic cortex	transfer of toxic substance(s) across specific synapses or the lack of trophic substance(s) necessary for neuronal survival
Zweig et al., 1988	The goal of the study was to detemine neuronal loss and NFTs presence within LC and DRN	25	12	Authors reported an average cell loss of DRN in AD of 64%; duration of AD correlated inversely with NFTs counts particularly within DRN; AD patients with major depression had fewer LC and DRN neurons in comparison with nondepressed patients	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed, together with sampling done only at the level of trochlear nucleus prevents drawing firm conclusions; data obtained imply a correlation between NFTs counts in DRN and rate of AD progression
Wilcock et al., 1988	The goal of the study was to determine DRN cell loss in AD	18	10	Authors reported an average loss of cell from DRN in AD patients of 33%	Biased methodology of estimation of nerve cell loss and frequency of NFTs from densities assessed, together with sampling done only at the level of trochlear nucleus prevents drawing firm conclusions
Hertz, 1989	A hypothesis was given that AD is an anterograde degeneration originating in the brainstem			A degeneration of adrenergic neurons in LC and serotoninergic neurons in RN may lead to impairment in metabolic and functional interactions between neurons and astrocytes	Author gave a conceptual framework for AD pathogenesis: termination of many of ascending adrenergic and serotonergic fibers in varicosities, from which released transmitter molecules reach their targets by diffusion, suggests their potential impact on both neuronal and glial cells, with eventual influence on their membrane transport, energy metabolism and glutamate release
Van Domburg, 1990	The goal of the study was to count the brainstem monoaminergic neurons in PD and AD	5	5	In comparison with controls, the author reported an average of 39.7% cell loss from DRN in AD patients	This is one of the first studies done with unbiased stereologic methods and throughout the whole rostrocaudal extent of DRN; the number of cases analysed was relatively low, but the amount of DRN cell loss was found to be substantial
Aletrino et al., 1992	The study was devoted to the development of an efficient and reliable sampling scheme to determine the total cell number in DRN using the optical fractionator method	8	10	The study showed severe cell loss of AD patients in the DRN of 39.4% and a tendency for cell shrinkage of the remaining cells.	With slightly higher number of cases analyzed, the methodology and results obtained are the same as the study of Van Domburg (1990)
Halliday et al., 1992	The goal of the study was to analyze the number of brain stem serotoninsynthesizing neurons AD patients and agematched controls using PH8 antibody	11	5	In comparison with controls, a large (70–80%) decrease in the number of PH8+ neurons in DRN and MRN of the AD+ subgroup (patients with significant AD-related pathology in RN) was found. In subgroup of AD-patients (those without pathology in RN), the reduction of PH8+ neurons was found only in DRN, but this change was not as dramatic as the „pure AD“ i.e. AD+ group	Authors concluded that RN are damaged only in certain cases of AD, indicating that, despite patient similarities in both clinical presentation and cortical neuropathology, the disease process is not homogeneous; the consistent and specific pathology of RN found in AD+ cases and the absence of such pathology in AD-cases suggested that pathology in RN is not essential and, therefore, causal for dementia: patients with earlier onset dementia had milder pathology in RN compared to later-onset patients

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Brilliant et al., 1992	The goal of the study was to determine distribution of β-amyloid in the brainstem	19 (13 AD and 6 AD+PD)	7	In AD patients the density of β-amyloid was highest in the midbrain (nucleus ruber and nucleus niger were spared), moderate in pons and scarce in the medulla	β-amyloid deposition is a function of synaptic connectivity, rather than passive diffusion from vascular sources
Lippa et al., 1996	The goal of the study was to determine does FAD and SAD cases differ neuropathologically	25 FAD (19 due to *PS-1* mut. + 6 due to *APP* mut.) and 11 SAD	none	There was no difference in the pattern of distribution of the neuronal loss, amyloid plaques, NFTs and NPs between FAD and SAD cases	Similarity of the neuropathological changes observed suggests a final common pathway for all forms of AD (however, FAD groups could be distinguished from SAD by the greater severity and the lack of influence of *APOE* genotype on pathology)
Mössner et al., 2000	The goal of the study was to review allelic variations in the serotonin transporter in AD and PD			Association studies showed that the low-activity allele of the transporter is the risk factor for late-onset AD	Compromized serotoninergic system may play an important role in the pathophysiology of AD
Parvizi et al., 2000, 2001	The goal of the study was to assess NFTs and SPs in PAG (Parvizi et al., 2000) and on serial sections from the entire brainstem (Parvizi et al., 2001) of AD and control subjects using thioflavin S-stained sections and immunocytochemis try using 10D5, ALZ-50 and AT8 antibodies	32	26 (13 from normal subjects and 13 non-AD patients)	In 72% of AD cases there were only SPs, while both SPs and NFTs were found in 9%. These changes were absent in all 26 control brains (Parvizi et al., 2000); different monoaminergic, cholinergic and other nuclei of the brainstem were found affected to contain SPs and NFTs with varying degrees of severity, whereas no changes were seen in the brainstems of controls (Parvizi et al., 2001)	Authors concluded that PAG is selectively involved in AD; furthermore, the type and density of pathological changes correlated with the duration of dementia (Parvizi et al., 2001); authors concluded that the finding of severe pathological changes in some brainstem nuclei raises the possibility that the dysfunction of these nuclei may contribute to cognitive defects and increased rates of morbidity and mortality in AD patients
Rüb et al., 2000	The goal of the study was to analyze AD-related cytoskeletal pathology in the raphe nuclei using modified silveriodide-Gallyas staining and the AT8 antibody	27		Examination of serial sections from the brainstems of 27 postmortem cases with BB I-VI of cortical cytoskeletal lesions showed that DRN manifests the cytoskeletal lesions early on (i.e. in cortical stages I-II), the central and linear RN do so in cortical stages III-IV while the caudal raphe nuclei only in cortical stages V-VI	Authors concluded that the developing damage within the nuclei of the raphe system correlates with the BB I-VI; as the source of the ascending serotoninergic system, the involvement of the oral raphe nuclei may be partially responsible for the early manifestations of the noncognitive and emotional deficiencies in AD
Yang and Schmitt, 2001	The goal of the study was to examine NCS, NRD and LC in FTLD, AD and non-demented controls	30 AD, 12 FTLD	35	The FTLD cases showed a significant, 40%, decline in number of neurons in the NCS and NRD, while the LC was spared; the magnitude of neuronal loss matched that of AD where, by contrast, the LC was also severely changed; β-amyloid deposition and NFTs occurred in the aminergic nuclei almost exclusively in AD and, only to a minor extent, in some aged controls	Authors concluded that the serotoninergic raphe nuclei with ascending projections to the forebrain, but not the LC, become directly or indirectly involved in FTLD both with and without motor neuron disease (MND)
Kovacs et al., 2003	One goal of the study was to compare the percentage of neurons synthesizing serotonin in NCS and NROP in AD and control brains	10	11	As compared with controls, the authors reported about 20% less neurons counted in NCS of AD subjects	Biased methodology of counting from densities observed and counting done only in 2 sections prevents drawing firm conclusions
Hendricksen et al., 2004	The authors hypothesized that greater NFTs pathology and fewer serotonergic neurons would be found in the DRN in	8 AD with depression, 7	14 elderly subject s with major	No differences in neuritic pathology or neuronal density were found between the subjects with depression and the	The authors concluded that if serotonergic dysfunction occurs in older depressed subjects, it is not due to neuronal loss in the

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	depressed compared to nondepressed elderly subjects and in AD patients with depression, compared to AD patients without depression	AD without depression	depression, 10 nondepressed elderly subjects	nondepressed controls; AD subjects taken altogether showed markedly fewer serotonergic neurons and associated higher levels of neuritic pathology, compared with subjects with primary depression and the nondepressed comparison subjects; however, AD subjects with with comorbid major depression did not differ from AD subjects without depression on these two measures	brainstem; biased methodology of counting from densities observed and sampling done using only 3 sections prevents drawing firm conclusions
Kepe et al., 2006	The goal of the study was quantification of 5-HT_{1A} receptor densities in the living brains of AD, MCI and control subjects using PET	8 AD, 6 MCI	5	AD patients had significantly decreased 5-HT_{1A} receptor densities in both hippocampi and raphe nuclei; when volume losses were included, the 5-HT_{1A} density losses were even more severe (average mean decrease of 24% in MCI and 49% in AD); these decreases correlated with decreased glucose utilization	The authors anticipated that the combined evaluation of 5-HT1a receptors densities together with [^{18}F]FDNNP PET (targeting NFTs and NPs) and [^{18}F]FDG PET (targeting neuronal function) offers a great opportunity for reliable, noninvasive detection of early neuropathological changes in AD
Bernedo et al., 2009	The goal of the study was to estimate the number of DRN and MRN serotonergic neurons using optical fractionator method in young and age dogs with and without β-amyloid deposits		8 young and 11 aged dogs	Aged dogs with β-amyloid cortical pathology had 33% fewer tryptophan-hydroxylase positive (TH+) serotonergic neurons in the DRN and MRN than aged dogs without β-amyloid cortical deposits; no significant variations were found between young and aged dogs without β-amyloid cortical deposits	Authors suggested that degeneration of the serotonergic neurons could be involved in the cognitive damage that accompanies β-amyloid cortical pathology in aged dogs
Grinberg et al., 2009	The goal of the study was to determine during which BB stage and how frequently the DRN is affected by AD-related neurofibrillary changes	80 (with BB ≥1)	38 (with BB = 0)	More than 20% of BB 0 and all of BB ≥ 1 cases were found to have substantial neurofibrillary changes in DRN	Authors concluded that their observations support the hypothesis of transneuronal spread of neurofibrillary changes from the DRN to its interconnected cortical brain areas; supratrochlear subnucleus of the DRN is affected by neurofibrillary changes prior to transentorhinal cortex during the disease process underlying AD

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonine); AD, Alzheimer's disease; *APOE, APOLIPOPROTEIN E* gene; *APP, AMYLOID PRECURSOR PROTEIN* gene; BB, Braak and Braak stage; CON, control cases; [^{18}F]FDG PET, positron emission tomography with 2-deoxy-2-[18F]fluoro-D-glucose as a radioligand; [^{18}F]FDNNP PET, positron emission tomography with 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene) malononitrile as a radioligand; FAD, familial AD; FTLD, frontotemporal lobar degeneration; DRN, dorsal raphe nucleus, LC, locus coeruleus; MCI, mild cognitive impairment; MND, motor neuron disease; MRN, median raphe nuclei; NCS, nucleus centralis superior; NFD, neurofibrillary degeneration; NFTs, neurofibrillary tangles; NROP, nucleus raphe obscurus and pallidus; PAG, periaqueductal gray matter; PH8, monoclonal antibody raised to phenylalanine hydroxylase, which cross-reacts with tryptophan hydroxylase, the serotonin synthesis enzyme; PD, Parkinson's disease; PET, positron emission tomography; *PS-1, PRESENILIN-1* gene; RN, raphe nuclei; SAD, sporadic AD; TH+, tryptophan-hydroxlase positive neurons