Circadian system rhythm disorders in aging and Alzheimer’s disease. Role of changes in melatonin, suprachiasmatic nucleus and corticosteroids

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CHAPTER 3

The first appearance of neurofibrillary tangles and plaques in the temporal cortex of aged controls is accompanied by decreased cerebrospinal fluid melatonin levels

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Abstract
Braak and Braak developed a system, now well-established, for staging the spread of neuropathological changes in Alzheimer's disease (AD). According to Braak's description the AD process starts in the temporal cortex. In order to evaluate the severity of AD neuropathology in the temporal cortex quantitatively, an additional modified Braak staging for the cortex (MBSC) was used in the present study. Melatonin is a hormone that does not only play a major role in the regulation of the circadian rhythms, but that may also exert neuroprotective effects in AD. Melatonin levels were determined in ventricular postmortem CSF of 44 cognitively unimpaired aged controls, 11 transition AD patients and 66 definite AD patients in relation to the neuropathological changes in the brain. We found that both the Braak stages of AD and the stages of the MBSC were negatively correlated with CSF melatonin levels. By using the MBSC stages the present study revealed for the first time that the first appearance of neurofibrillary tangles and neuritic plaques in the temporal cortex of controls is accompanied by decreased melatonin levels. Those controls that did not have any neurofibrillary tangle or neuritic plaque in the temporal cortex, had 3-7 times higher melatonin levels than the ones with a few neurofibrillary tangles and a few neuritic plaques in the temporal cortex. Such a significant relationship was not found for the frontal, parietal or occipital cortex in controls. No significant correlation was found between ventricular CSF-melatonin levels in aged controls with or without any AD pathology changes on the one hand, and brain weight, postmortem delay (PMD) or pH of CSF on the other. We conclude that the decrease of CSF melatonin levels may be an early event in the development of AD, possibly occurring even before any clinical symptoms become overt.
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Introduction

Melatonin is a hormone that does not only play a major role in the regulation of circadian rhythms (1), but also functions as part of an anti-oxidative defense system (2). Oxidative stress has been proposed to play a part in the pathogenic mechanisms of Alzheimer's disease (AD) (3). Increased expression of anti-oxidative enzymes and heat-shock proteins are key markers of oxidative stress. The observation that such proteins are present in the neuropathological lesions of AD patients supports the possible involvement of oxidative stress in pathogenic mechanisms in this disorder (4-6). Deposition of cerebral amyloid in senile plaques is a major neuropathological hallmark of AD. It is, therefore, of great interest that, in vitro, melatonin protects neurons against oxidative stress and β-amyloid toxicity (7). Recently, we found a dramatic decrease in cerebrospinal fluid (CSF) melatonin levels with increasing age, and even more so in AD patients (8). The decrease of nocturnal melatonin secretion is related to mental impairment (9). It has, therefore, been proposed that the gradual loss of melatonin levels in advanced age (10,11) and even more so in AD (8,12) might contribute to the AD process (13). As the brain is presumed to be the main target for melatonin actions, the reduction in melatonin levels in AD may contribute to the exposure of vulnerable brain areas to free radicals, β-amyloid and other toxic compounds. We hypothesized that advances in neuropathological stages would be associated with a decline in melatonin levels. In the present study, we evaluated the relationship between the Braak stages that indicate the spread of AD changes over the brain and a modified Braak score (MBSC), indicating the severity of the AD stages in the cortex on the one hand, and the levels of melatonin in postmortem CSF on the other.

Materials and methods

Subjects

Autopsies were performed within the framework of the Netherlands Brain Bank following permission for a brain autopsy and the use of tissues and clinical information for research purposes. Ventricular postmortem CSF was obtained at autopsy, generally between 1 to 12h after death, from 66 definite AD patients, 11 transition AD patients and 44 controls without a neurological or psychiatric disease. The charts of the control subjects did not report any psychiatric or neurological disease. The controls were all at Braak stage 0-II. The definite AD patients fulfill the NINCDS-ADRDA criteria (14), i.e., they had a clinical diagnosis of probable AD excluding other causes of dementia by means of history, physical examination and laboratory tests and a neuropathological diagnosis with Braak’s stages V-VI. The transitional AD patients were those who fulfilled the NINCDS-ADRDA criteria of probable AD and had a Braak’s stage III-IV. Before the brain was removed, ventricular CSF was collected and pH was determined immediately as a
Table 1 Brain material

<table>
<thead>
<tr>
<th>Group</th>
<th>Number (male/female)</th>
<th>Age (years)</th>
<th>Brain weight (grams)</th>
<th>PMD (hours)</th>
<th>CSF pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44 (24/20)</td>
<td>76±2</td>
<td>1237±20</td>
<td>7.5±0.6</td>
<td>6.7±0.05</td>
</tr>
<tr>
<td>TAD</td>
<td>11 (1/10)</td>
<td>87±1</td>
<td>1098±43</td>
<td>3.9±0.4</td>
<td>6.8±0.10</td>
</tr>
<tr>
<td>AD</td>
<td>66 (25/41)</td>
<td>77±2</td>
<td>1137±21</td>
<td>4.2±0.1</td>
<td>6.6±0.03</td>
</tr>
</tbody>
</table>

Notes: PMD: postmortem delay; CSF: cerebrospinal fluid; Control: subjects with Braak stage 0-II; TAD: transition Alzheimer's disease with Braak stage 3-4; AD: Alzheimer's disease with Braak stage V-VI.

Measure of agonal state (15). CSF was immediately centrifuged at 700g. The supernatant was subdivided into 250-1000μl aliquots that were kept at -80°C until assayed. The following variables were included in the present study, both for AD patients and controls: postmortem delay (PMD), clock time of death, month of death, CSF-pH, brain weight, sex, and age (Table 1). The severity of dementia was evaluated by Reisberg's global deterioration scale (16).

Neuropathology evaluation

All brains were investigated in a systematic way by a neuropathologist (Prof. F. C. Stam, The Netherlands Brain Bank, Dr. W. Kamphorst, Free University Amsterdam, or Prof. D. Troost, Academic Medical Center, University of Amsterdam). After a standard fixation time of four weeks one neuropathologist (WK) estimated the distribution of the AD changes over the brain according to the stages of Braak and Braak (17). Moreover, in order to give a measure for the severity of AD changes per cortical area, a modified Braak score of the amount of neurofibrillary tangles (NFT), neuritic plaques (NP) and disruption of the neuropil (DN) was performed for the cortex (MBSC) in formalin-fixed paraffin-embedded sections. Bodian stained sections of the frontal, temporal, parietal and occipital cortex were included. In each cortical area of the AD patients and controls changes were separately scored as 0 = absent, 1 = present but less than moderate, 2 = moderate (i.e. two or three neurofibrillary tangles, two or three neuritic plaques or 30-60% of the normal network replaced by neuropil threads per 0.4 mm²) and 3 = more than moderate (Fig. 1). For example, TNP=0 means that there were no neuritic plaques found in the temporal cortex. TNP=1 means that plaques were present but in amounts lower than two or three neuritic plaques per 0.4 mm². TNFT=0 means that there were no neurofibrillary tangles present in the temporal cortex. TNFT=1 means that tangles were present but in amounts lower than two or three neurofibrillary tangles per 0.4 mm². The maximum possible score of MBSC means is the summation of all scores in all 4 cortical areas examined, i.e., 4 x (3+3+3)=36, a score that was only reached in severe Alzheimer's cases.
Fig. 1 A modified Braak staging in the cortex (MBSC) in Bodian stainings of the temporal cortex in formalin-fixed tissue. Changes in AD patients and controls were separately scored as 0= absent (A,E); 1= present but less than moderate (B,F); 2= moderate (i.e. 2 to 3 neurofibrillary tangles (NFTs), 2 to 3 neuritic plaques (NPs) per 0.4 mm$^2$ or 30-60% of the normal network replaced by neuropil threads (C,G); and 3= more than moderate (D,H). Arrows indicate the NPs or NFTs. Scale bar=50μm.
**Melatonin assay**

Melatonin in postmortem CSF was measured by a direct radioimmunoassay. The assay was run in a 0.1 mol/l tricine buffer (Sigma Chemicals) containing sodium chloride (0.15 mol/l, Merck) and 0.1% gelatin (Merck) adjusted to pH=7.5. Iodinated melatonin ($^{125}$I) (Amersham IM215, Roosendaal, The Netherlands) was diluted in tricine buffer, at a final concentration of 25000 cpm/ml. The melatonin antibody (AB/R/O3, Stockgrand, Guildford, UK) that was raised in rabbits was shown to be sufficiently specific for clinical application in CSF without pre-assay treatment. The antibody cross-reacted with 6-hydroxymelatonin at 5.3% and at less than 0.2% with 6-sulphatoxymelatonin (18). Standards were diluted in tricine buffer in a range of 1 pg/ml to 1000 pg/ml. Samples of CSF (100μl) were aliquoted in tubes with 100μl tricine buffer and 250μl anti-melatonin (final dilution 1:200,000). They were vortexed, capped and incubated for three nights at 4°C. Bound melatonin was separated by 50μl donkey anti-rabbit antiserum coupled to cellulose (SAC-CEL, IOS, Boldon, UK). The precipitate was counted in a gamma Counter (Cobra 500s, Packard, Groningen, The Netherlands). The intra-assay coefficient was 8.7%.

**Statistical Analysis**

Differences in melatonin levels between groups were tested using a Mann-Whitney U-test. The effects of sex, age, postmortem delay, brain weight, clock time of death, pH and neuropathological changes in controls and AD patients on CSF melatonin levels were evaluated statistically by multiple regression. The level of significance was 0.05 for all tests.

**Results**

**Correlation between melatonin levels and stages of neuropathy**

When testing eight variables, i.e. Braak's stages of AD, MBSC stages of AD, clock time of death, postmortem delay, brain weight, month of death, sex and age and (stepwise) multiple regression analysis revealed that only the Braak's stages of AD and MBSC stages of AD were negatively correlated with CSF melatonin levels. ($r=-0.33, P<0.004$; $r=-0.35, P<0.0001$ respectively) (Fig 2, Fig 3).

**Temporal cortex AD changes are correlated to decreased melatonin levels in aged controls by MBSC evaluation**

In order to see in which cortical area the severity of the neuropathological changes was the most strongly related to the decreased melatonin levels, MBSC staging was used for further analysis. On the one hand we found a negative correlation between the CSF melatonin levels and the MBSC stages in the temporal cortex ($r=-0.33, P=0.03$), and not in
Fig. 2 Relationship between Braak's stages and melatonin levels in cerebrospinal fluid (CSF). Note that the decrease in CSF melatonin levels started in the aged control subjects who had the first signs of Alzheimer pathology (Braak stage II).

Fig. 3 Relationship between the modified Braak staging in the cortex (MBSC) and the melatonin levels in cerebrospinal fluid (CSF). The amount of neurofibrillary tangles, neuritic plaques and disruption of the neuropil was scored in the frontal, temporal, parietal and occipital cortex in formalin-fixed (6 μm) sections. The maximum possible total score is 36 (see text). The MBSC range was subdivided into 7 classes. Note that there is already a dramatic decrease in CSF melatonin in the aged control subjects who had the first signs of neuropathological changes (Score 1-6).
the frontal, parietal or occipital cortex in controls (Braak stage 0-II). On the other hand, there was no correlation between the CSF melatonin levels and the MBSC stages in the temporal cortex (P=0.86) in AD patients (Braak stages V-VI).

The first appearance of NFT and NP in the temporal cortex in aged controls is accompanied by decreased melatonin levels.

In control subjects (Braak stage 0-II), CSF melatonin levels were 7 times higher in the group that had no neuritic plaques in the temporal cortex (TNP=0) (280.4±64 pg/ml; n=28), than in the group where a few neuritic plaques were found in the temporal cortex (TNP=1) (38.5±8 pg/ml; n=8; P=0.004). Furthermore, CSF melatonin levels were 3 times higher in the group in which no neurofibrillary tangles were found in the temporal cortex (TNFT=0) (287.3±68 pg/ml; n=26), than in the group that had a few neurofibrillary tangles in the temporal cortex (TNFT=1) (82.1±4 pg/ml; n=14) (P=0.02) (Fig 4). Such significant differences were not found in the frontal, parietal or occipital cortex in controls.

Fig. 4 Relationship between melatonin levels in CSF of aged control subjects and the neuropathological changes in the temporal cortex with MBSC. TNP=0 means that there were no neuritic plaques found in the temporal cortex. TNP=1 means that plaques were present but in amounts lower than 2-3 neuritic plaques per 0.4 mm² or 30-60% of the normal network were replaced by neuropil threads in the temporal cortex. TNFT=0 means that there were no neurofibrillary tangles present in the temporal cortex. TNFT=1 means that tangles were present but in lower amounts than 2-3 neurofibrillary tangles per 0.4 mm² or 30-60% of the normal neural network was replaced by neuropil threads in temporal cortex per 0.4 mm².
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CSF melatonin levels in relation to the PMD, pH of CSF and brain weight in aged controls

No significant correlation was found between CSF-melatonin levels in aged controls with early AD pathology changes and without any AD pathology changes on the one hand, and brain weight (r=-0.15, P=0.36; r=-0.18, P=0.35, respectively), PMD (r=-0.00018, P=0.98; r=-0.0001, P=0.98, respectively), or pH of CSF (r=-0.06, P=0.49; r=-0.09, P=0.48, respectively), on the other. In addition, there was no age difference, either between the subjects with TNFT=0 and TNFT=1 (P=0.65), or between the subjects with TNP=0 and TNP=1 (P=0.68).

Discussion

Neurofibrillary tangles, neuritic plaques and neuropil threads are the characteristic neuropathological hallmarks of AD. Braak's stages of both the amyloid-deposit and the neurofibrillary pathology indicate the extension of the neuropathological changes over the brain (17). In the present study, forward stepwise regression analysis revealed that the Braak's stages for tangles were negatively correlated with CSF melatonin levels. According to Braak's observations the AD process starts in the temporal cortex. In order to evaluate the severity of AD neuropathology in the cortex quantitatively, an additional modified Braak staging (MBSC) was used in the present study. The MBSC stages provide a detailed indication of the amount of AD changes per cortical area and a significant negative correlation was found between CSF melatonin levels and the MBSC stages. Both findings are in agreement with the hypothesis that melatonin may be a factor involved in the pathogenesis of AD neuropathology, although other explanations for these correlations are also possible.

Neuronal damage as a result of oxidative stress has long been considered as a critical mechanism of cellular damage, not only in normal aging (20), but also in neurodegenerative diseases (21,22). Emerging evidence indicates that oxidative stress may be involved in the pathogenesis of the typical neuropathological hallmarks of AD. Pappolla (4) found a subgroup of neurofibrillary tangles (15%-25%) and senile plaques (50%) that showed immunoreactivity for both superoxide dismutase (CuZn-forms and Mn-forms) and catalase. Tangle-free neurons in both diseased and control brains showed weak to absent intracytoplasmic immunoreactivity for these enzymes. Good et al (23) demonstrated the presence of nitrotyrosine in neurofibrillary tangles of AD, and its absence in controls lacking neurofibrillary tangles. Melatonin not only neutralizes oxygen-derived free radicals, but can also scavenge other types of carbon-centered free radicals (24). In addition, melatonin effectively reduces the lipid peroxidation induced in vitro by β-amyloid or aluminum (25), while oxidative damage of mitochondrial DNA caused by β-amyloid in vitro was also prevented by melatonin (26). In contrast with conven-
tional anti-oxidants, melatonin secretion is significantly decreased in aging humans and AD patients (8,12,27). One may therefore hypothesize that the high levels of ventricular fluid melatonin in intact controls may protect the brain against hydroxyl radical damage, especially as far as periventricular metabolically active neural tissue is concerned (28), and that this protective mechanism fails in AD. In this connection it may be of importance that in sheep melatonin levels in the third ventricle are 7-fold higher than those in the lateral ventricle (29), while the hypothalamus of AD patients shows relatively few silver staining neurofibrillary tangles and neuritic plaques (30). A second possible mechanism for the relationship between the decreased melatonin levels and the development of AD neuropathology may be the effect of melatonin on amyloid metabolism. Melatonin protects neurons against β-amyloid toxicity in vitro. Melatonin interacts with β-amyloid 1-40 and β-amyloid 1-42 and inhibits the progressive formation of the β-sheets of the amyloid fibrils (4,7). Interestingly, in the present paper, by using staging according to the MBSC system, we found that the occurrence of the first AD changes in the temporal cortex, where the AD process starts (17), is correlated to diminished melatonin levels. Those controls that did not have any NFT or NP had much higher CSF melatonin levels than the controls that had a few NFTs or NPs in the temporal cortex. This finding was not due to a difference in age, sex or the clock time of death between the two groups. Moreover, in the other cortical areas, i.e. frontal, parietal and occipital, we did not find such a relationship between decreased CSF melatonin levels and the occurrence of neuropathological changes. A number of studies have demonstrated that the temporal structures, the hippocampus and entorhinal cortex, are indeed the first areas affected by neurofibrillary degeneration and that neurofibrillary degeneration in these areas is associated with the first signs of cognitive decline (17). Our data therefore point to the possibility that the temporal cortex is the most vulnerable cortical area to the decreased CSF melatonin levels in AD. In an earlier study, we found strongly decreased CSF melatonin levels in aging and AD. The amount of decrease in CSF melatonin levels was related to the presence of an ApoE-ε4 genotype (8), which is known to be one of the major risk factors for the senile form of AD. In AD ApoE-ε4 genotype also goes together with a diminished metabolic rate in the tempo-parietal region (31). Decreased neuronal metabolism is one of the hallmarks of AD and probably crucial in the development of dementia (32).

A possible explanation for our observations would be that melatonin production decreases when early AD changes appear in controls. Indeed, pineal calcification increases during the process of aging (33) while day-night fluctuations of the pineal melatonin content and melatonin production decrease (12). However, according to Pardo et al. the pineal parenchyma is not affected very early in the AD process. On the other hand, the noradrenergic fibers of the pineal are affected in AD, possibly resulting in a reduction in melatonin production (34). Whether this occurs already in controls in the earliest phase
of the disease process has to be investigated. A possible confounder for the observed decrease in CSF melatonin levels would be a strong increase in CSF volume or an increase in CSF turnover between Braak stages 0 and II or between the total neuropathology scores 0 and I-VI. It has been shown that ventricular CSF is increased by 99% in late stages of AD (35,36) and also in aging, by 40% (37). However, there are no data available on the volume changes in CSF in the very early AD stages in aged controls where we found the strong decrease in melatonin levels. Pleading strongly against the possibility of a dilution of CSF are the observations that the CSF fluid production rate is decreased and the total protein concentrations in CSF are higher in the elderly than in young individuals (38,39). In a three-year follow-up of CSF tau and β-amyloid-42 concentrations in AD, a decrease of CSF Aβ-42 was found with time while no consistent changes in CSF tau levels were found (40). In addition, we found that the levels of the adrenal hormone cortisol in CSF of senile AD patients did not differ from those of aged controls (41), which also pleads against a dilution of CSF.

The finding of a significant negative correlation between low melatonin levels and the earliest neuropathological changes in temporal cortex of controls are of particular interest because they may provide information about the very first stages of the disease that could not be monitored in vivo until now by any other means. The possibility that a decrease in melatonin levels may be a marker for very early AD changes should be confirmed in plasma or saliva. A long-term follow-up of aged controls and their saliva melatonin levels up to the moment they develop the first cognitive complaints seems to be a realistic possibility.

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