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

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Decreased melatonin levels in postmortem cerebrospinal fluid in relation to aging, Alzheimer’s disease and ApoEε 4/4 genotype

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Sleep disruption, nightly restlessness, sundowning and other circadian disturbances are frequently seen in Alzheimer’s disease (AD) patients. Changes in the suprachiasmatic nucleus and pineal gland are thought to be the biological basis for these behavioral disturbances. Melatonin is the main endocrine message for circadian rhythmicity from the pineal. In order to see whether melatonin production was affected in AD, melatonin levels were determined in the cerebrospinal fluid (CFS) of 85 patients with Alzheimer's disease (AD) (mean age = 75±1.1 years) and in 82 age-matched controls (mean age = 76±1.4 years). Ventricular postmortem CSF was collected from clinically and neuropathologically well-defined AD patients and from control subjects without primary neurological or psychiatric disease. In old control subjects (over 80 years of age) CSF melatonin levels were half of those in control subjects of 41-80 years of age (176 ± 58 pg/ml, n=29 and 330 ± 66 pg/ml, n=53 respectively; P = 0.016). We did not find a diurnal rhythm in CSF melatonin levels in the control subjects. In AD patients the CSF melatonin levels were only one fifth (55±7 pg/ml) of those in control subjects (273±47 pg/ml) (P=0.0001). There was no difference in the CSF melatonin levels between the presenile (42±11 pg/ml; n=21) and senile AD patients (59 ± 8 pg/ml; n=64) (P=0.35). The melatonin level in AD patients expressing APOEε 3/4 (71 ± 11 pg/ml) was significantly higher than that in patients expressing ApoEε 4/4 (32±8 pg/ml) (P=0.02). In the AD patients no significant correlation was observed between age of onset or duration of AD and CSF melatonin levels. In the present study, a dramatic decrease in the CSF melatonin levels was found in old control subjects and even more so in AD patients. Whether supplementation of melatonin may indeed improve behavioral disturbances in AD patients should be investigated.
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Introduction

The circadian rhythm of melatonin secretion is generated in the suprachiasmatic nucleus (SCN) (1). In previous studies, a decreased number of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) neurons in the SCN was found during aging and even more dramatically so in Alzheimer’s disease (AD) (2,3). In addition, an impaired daily variation in the concentration of melatonin in the human pineal gland was found in the older subjects and even more so in AD patients (4). Changes in the SCN and pineal gland are considered to be responsible not only for the disturbed circadian rhythms in hormones, body temperature and sleep-wake behavior, but also for behavioral disorders in elderly people and AD patients. Demented patients often suffer from sleep disruption, nightly restlessness and sundowning (5).

Disruption of sleep of the care giver due to nocturnal restlessness of the patient is a more important reason for placement of a demented patient in a nursing home than cognitive impairment (6). Moreover, disturbed circadian rhythms are considered to be related to the cognitive performance of elderly people and AD patients(7,8). In addition, it was reported that melatonin inhibits the progressive formation of β-sheets and amyloid fibrils in vitro (9).

Although various studies indicate that the circadian rhythm of melatonin is disturbed during aging (10-14), only limited information on serum melatonin in dementia is available (8,15) and information on melatonin levels in cerebrospinal fluid (CSF) is totally lacking. Since the brain is presumed to be the main target for melatonin action, we determined in the present study melatonin levels in postmortem CSF during aging and in neuropathologically confirmed AD patients.

Material and methods

Autopsies were performed within the framework of the Netherlands Brain Bank. Ventricular postmortem CSF was obtained at autopsy, 1-12h after death, from 85 Alzheimer patients and 82 controls without a primary neurological or psychiatric disease. Before the brain was removed, ventricular CSF was collected and pH was determined immediately as a measure of agonal state. Individuals who die after a long terminal phase accumulate lactic acid and have, therefore, a lower pH(16) independent of the postmortem time (17). CSF was immediately centrifuged at 700g. The supernatant was subdivided into 250-1000 µl aliquots that were kept at -801 C until assayed. The following variables were included in the present study for both Alzheimer patients and controls: postmortem interval, CSF-pH, brain weight, sex, age, clock time and month of death (Table I). All Alzheimer patients had a history of a gradual intellectual deterioration, and the diagnosis “probable Alzheimer’s disease” was made according to the NINCDS-ADRDA criteria (18) excluding other causes of dementia by means of history, physical examination and
Table 1 Clinical and pathological data for the controls and Alzheimer's patients studied

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>AD group</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases (m/f)</td>
<td>82 (44/38)</td>
<td>85 (33/52)</td>
<td>0.06</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>74.71 ± 1.2</td>
<td>76.26 ± 1.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1215 ± 15</td>
<td>1056 ± 17</td>
<td>0.001</td>
</tr>
<tr>
<td>Postmortem delay (h)</td>
<td>6.91 ± 0.36</td>
<td>4.21 ± 0.12</td>
<td>0.001</td>
</tr>
<tr>
<td>PH of CSF</td>
<td>6.71 ± 0.05</td>
<td>6.59 ± 0.03</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM

laboratory tests. Severity of dementia was evaluated by the global deterioration scale (GDS) (19). All brains were investigated in a systematic way by neuropathologists (Prof. F.C. Stam, the Netherlands Brain Bank, Dr. W. Kamphorst, Free University, Amsterdam or Dr. D. Troost, Academic Medical Center, University of Amsterdam). The neuropathological diagnosis 'Alzheimer's disease' was made on the basis of the occurrence of many senile plaques, neurofibrillary tangles and a disorganized fiber pattern, the presence of dystrophic neuritis and neuropile threads in Bodian and Congo stainings of the hippocampus and 5 cortical areas in formalin-fixed tissue (20). To exclude the presence of Parkinson's disease, the substantia nigra was also examined. In order to determine whether diurnal variations were present in levels of melatonin, subjects were grouped into two diurnal periods based on the clock time of death: 1000-2200 h and 2200-1000 h, since these times are known to be associated with circadian differences in the level of melatonin in human plasma (21-24). We also checked whether there was any correlation between the season of death and CSF melatonin levels in controls and AD patients. Subjects were divided into 4 seasonal groups based on the date of the death: i.e. spring (21st of March - 21st of June), summer (21st of June - 21st September), autumn (21st of September - 21st of December) and winter (21st of December - 21st of March).

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 (2-[125] Iodomelatonin, 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 (25). Standards were diluted in tricine buffer to give a range of dilutions from 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 4EC. Bound melatonin was separated by 50 μl of donkey anti-rabbit antiserum coupled to cellulose (SAC-CEL, IOS, Boldon, UK). Precipitate was counted in gamma Counter (Cobra 500s, Packard, Groningen, The Netherlands). Intraassay coefficient was 8.7%.

ApoE assay
ApoE genotyping was performed on frozen tissue from the cerebellum of the AD patients. The genotype of each extracted DNA sample was determined by PCR amplification using the primers 5'TCCAAGGAGCTGCAGGCGGCGCA3' and 5'ACAGAATTCGCCCCGGCCTGGTACACTGCCA3'. Then the PCR product was digested by CfoI and fragments were separated by electrophoresis in a 5% agarose gel.

Statistics
Differences in melatonin levels between groups were tested using the Mann-Whitney U-test. The difference in number of males and females between controls and AD patients was tested by Chi-square. The effects of sex and postmortem time on CSF melatonin levels were evaluated statistically by a two-factor analysis of variance (ANOVA). Correlations of postmortem interval, brain weight and pH of CSF versus melatonin levels were analyzed by the Spearman correlation test. Difference among three groups was tested by Kruskal-Wallis ANOVA. All results were expressed as means±standard error of the mean (SEM). Differences were considered statistically significant at the P<0.05(two-tailed) level.

Results
A larger brain weight was found in the controls as compared to the AD patients (1215±15 g vs 1056±17 g , P=0.001). In AD patients the melatonin levels (56±7 pg/ml) were five times lower than those in controls (273±47 pg/ml, P=0.0001) (Fig. 1). Presenile AD patients (<65 years of age, n=21) had decreased CSF melatonin levels (42±11 pg/ml) which were 5 times lower than those in young controls (254±75 pg/ml, n=12, P=0.01). The melatonin levels of presenile AD patients (42 ± 11 pg/ml) did not differ from those of senile AD patients(59±8 pg/ml, P=0.35). The difference between senile AD patients (n=64) and controls older than 65 years of age (270±54 pg/ml, n=70) were significant (P=0.0001). There was no difference in CSF melatonin according to the severity of dementia: the CSF melatonin in AD patients with GDS 7 (57±9 pg/ml, n=50) did not differ from those of GDS 6 (53±11 pg/ml, n=18) or from those of GDS less than 6 (33±11 pg/ml, n=9) ( P=0.82). No significant correlation was found between age at onset of the
dementia and CSF melatonin levels in AD patients \( r=0.07, P=0.52 \). In addition, no correlation was observed between duration of AD and CSF melatonin levels \( r=-0.10, P=0.37 \).

In controls a significant decrease in ventricular CSF-melatonin was found with age. Melatonin levels of controls older than 80 years of age \( (176\pm58 \text{ pg/ml, n=29}) \) were found to be 50% lower than those of controls who were of 41-80 years \( (330\pm66 \text{ pg/ml, n=53, P=0.016}) \) (Fig. 2). No significant daily rhythm in CSF melatonin levels was detected in

![Graph 1](image1)

**Fig. 1** Melatonin levels in cerebrospinal fluid (CSF) of control subjects (n=82) and Alzheimer's disease (AD) patients (n=85). *: P<0.0001.

![Graph 2](image2)

**Fig. 2** Melatonin levels in cerebrospinal fluid (CSF) of control subjects of 41-80 years of age (n=53) and older than 80 years (n=29). *: P<0.01.
Fig. 3 Melatonin levels in cerebrospinal fluid (CSF) of control subjects and Alzheimer's disease (AD) in relation to the clock time of death. Note that the overall levels of melatonin in AD were significantly lower than those in controls and that there was no obvious day/night rhythm in CSF melatonin levels, either in controls or in AD.

Fig. 4 Melatonin levels in cerebrospinal fluid (CSF) of control subjects and Alzheimer's disease (AD) in relation to the month of death. Note that there was no significant seasonal rhythm in CSF melatonin levels, either in controls or in AD.
AD patients (P=0.58) or controls (P=0.66). In controls, the nighttime level of CSF melatonin (269±52 pg/ml, n=44) was similar to that during the day (1000-2200h) (276±84 pg/ml, n=38) (Fig. 3). Two-way analysis of variance revealed that postmortem delay and sex had no effect on CSF melatonin levels in control subjects (P=0.18 and P=0.89 respectively). There was no difference in CSF melatonin levels between the different seasons in control subjects (spring: 157±54 pg/ml, n=18; summer: 234±46 pg/ml, n=21; autumn: 262±70, n=24; winter: 321±99, n=19; P=0.82; ANOVA) (Fig. 4).

An interesting finding of the present study was that there was a significant difference between AD patients expressing ApoE-ε 3/4 (n=32) and ApoE-ε 4/4 (n=17) in CSF melatonin levels (71±11 vs. 32±8 pg/ml, respectively P=0.02) (Fig. 5). There was only one control subject expressing ApoE-ε 4/4 genotype.

No significant correlation was found between ventricular CSF-melatonin levels in controls or AD patients on the one hand, and brain weight (r=-0.11, P=0.32; r=0.04, P=0.72, respectively), postmortem delay (r=-0.0002, P=0.99, r=-0.16, P=0.15, respectively), or pH (r=0.19, P=0.08; r=-0.08, P=0.47, respectively) on the other. There is, consequently, no reason to presume that the differences in brain weight, postmortem delay and pH of the CSF between controls and AD patients (Table 1) influenced the results in any way.
Discussion

The present study shows markedly lower melatonin levels in ventricular CSF of AD patients. Melatonin levels were 5-fold lower in AD patients than in age-matched controls. Interestingly, the amount of decreased nocturnal melatonin level was reported to be related to the severity of mental impairment in demented patients (8,27). The data in the literature concerning melatonin levels in dementia are, however, discordant. No differences in plasma or pineal melatonin levels between demented and elderly subjects were reported in earlier studies (4,10,27). Magri et al (8) found also no difference in plasma melatonin levels of 6 demented patients compared with normal elderly subjects. However, more recent studies showed a decrease in nocturnal plasma melatonin levels in senile AD patients (8,28). In addition, decreased pineal melatonin levels were found in aging and AD(4). The discrepancies between the studies on melatonin levels may be attributed to differences in the age of subjects, to the use of in- or outpatients, or to severity and type of dementia that also varied across studies. The subjects used in the present study were neuropathologically confirmed AD and control subjects. Our findings on the decreased CSF melatonin levels suggest that melatonin may indeed be involved in symptoms of AD. We did not find a relationship between the postmortem CSF melatonin levels and the onset, duration or severity of dementia. Others found also no relationship between the duration of dementia and the flattening of the melatonin rhythm in living demented patients (28). The decreased CSF melatonin levels observed by us coincide with the general disturbance of circadian rhythms, in AD e.g. in sleep-wake, body temperature and rest-activity(7) and with the degeneration of the SCN in aging and AD (2,29,30). Furthermore, demented patients tend to be exposed to less environmental light than healthy people (31). It has been reported that bright light therapy, an interference presumed to stimulate the SCN directly, was effective for sleep and behavior disorders in elderly patients with dementia (28,32). These observations support the idea that degeneration of the SCN in AD is the central phenomenon in these changes.

The observed decrease of ventricular CSF-melatonin levels with aging in controls supports other reports on plasma melatonin changes (33,34). Age of the subject had a significant effect on the day/night variation of pineal melatonin level, the rhythmicity being lost in the older age group (4). The decline in the production of melatonin with age agrees with previous reports (4,12,35–37), while in the older age group also SCN changes were observed (2).

It is proposed that the response of the circadian system to environmental (Zeitgeber) signals diminishes with aging, and that when the melatonin rhythm deteriorates during aging, other circadian rhythms likewise weaken and become desynchronized (38). Concerning the changes of plasma melatonin observed in elderly people, the mechanism responsible for the reduction of melatonin secretion in aging is not very well understood. Alterations in SCN (2,4) may be a major factor. Interestingly, a significant de-
crease of CSF melatonin was found in the control subjects who were older than 80 years. The decreased number of AVP neurons in the SCN was also found in the subjects older than 80 years (2), suggesting that the changes in the SCN and pineal are related. Structural changes of the pineal, such as the calcifications or the variations of melatonin clearance do not seem to play an important role in the decrease of plasma melatonin levels in elderly subjects (14,39). Nocturnal melatonin secretion is modulated by noradrenalin through β-receptors (40). Therefore it may be of importance that an impairment of cat-
echolaminergic pathways occurs with aging in the CNS[41]. The effect of a decline in the CSF production rate or turnover with aging (42-44) on CSF-melatonin levels in aging and Alzheimer's disease is not known.

In the present study a daily rhythm of melatonin levels in postmortem ventricular CSF was not observed in controls or AD patients. This may well be due to the fact that our CSF samples were obtained postmortem from hospitalized patients. It has been reported that hospitalized patients have significantly higher daytime plasma melatonin levels, an earlier nocturnal rise, and a more variable timing of their secretion profiles (15,45). Possibly artificial and supplementary natural lighting in the hospital may not be sufficient to suppress melatonin secretion adequately during daylight hours nor act efficiently to entrain day/night secretion of melatonin in a physiological circadian manner. This problem may exist particularly in humans. Room light of low intensity, which is sufficient to suppress melatonin secretion in other mammals, failed to do so in humans(46). Another reason for the lack of an overall circadian rhythmicity in CSF levels of melatonin may be that in spite of the reproducible pattern observed from day to day in the same individual, a very large interindividual variation was observed (47-48). In our study only one data point per patient was available for obvious reasons. In addition, a great variety of pathological conditions and disease states have been associated with alterations in pineal function and 24 hour melatonin profiles(4,11,49). So the normal range for daytime and night time plasma and CSF levels is very large, and the day-night difference for melatonin levels can vary widely for various reasons.

Recent studies have indicated a significant association between the ApoE type and AD. Apo E is a 34-KDa protein that plays a key role in the regulation of the metabolism of lipids and has three major isoforms (E2, E3 and E4). The ApoE-ε4 genotype is a risk factor for AD (50-52) and it is likely that this will to some degree be reflected in the neuropathology and neurochemistry of this disease. Indeed, ApoE immunoreactivity has been found in senile plaques and cerebral vessels and neurofibrillary tangles in AD. An interesting finding of the present study is that CSF-melatonin levels from ApoEɛ3/4 genotype patients were significantly higher than those from ApoEɛ 4/4 genotype, suggesting again a relationship between melatonin levels and signs and symptoms of AD.
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Acknowledgments

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