

Molecular Changes Underlying Reduced Pineal Melatonin Levels in Alzheimer Disease: Alterations in Preclinical and Clinical Stages

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A disturbed sleep-wake rhythm is common in Alzheimer disease (AD) patients and correlated with decreased melatonin levels and a disrupted circadian melatonin rhythm. Melatonin levels in the cerebrospinal fluid are decreased during the progression of AD neuropathology (as determined by the Braak stages), already in cognitively intact subjects with the earliest AD neuropathology (Braak stages I-II) (preclinical AD). To investigate the molecular mechanisms behind the decreased melatonin levels, we measured monoamines and mRNA levels of enzymes of the melatonin synthesis and its noradrenergic regulation in pineal glands from 18 controls, 33 preclinical AD subjects, and 25 definite AD patients. Pineal melatonin levels were highly correlated with cerebrospinal

fluid melatonin levels. The circadian melatonin rhythm disappeared because of decreased nocturnal melatonin levels in both the preclinical AD and AD patients. Also the circadian rhythm of β_1 -adrenergic receptor mRNA disappeared in both patient groups. The precursor of melatonin, serotonin was stepwise depleted during the course of AD, as indicated by the up-regulated monoamine oxidase A mRNA and activity (5-hydroxyindoleacetic acid:serotonin ratio). We conclude that a dysfunction of noradrenergic regulation and the depletion of serotonin by increased monoamine oxidase A result in the loss of melatonin rhythm already in preclinical AD. (*J Clin Endocrinol Metab* 88: 5898–5906, 2003)

SLEEP-WAKE RHYTHM DISRUPTION and other circadian disturbances are commonly seen in Alzheimer disease (AD) patients (1) and are the most frequent reason for nursing home placement, in fact more often than cognitive impairment (2). The circadian disturbances in AD are accompanied by decreased melatonin levels and a disrupted circadian melatonin rhythm (3–5). In addition to melatonin's ability to regulate circadian rhythms (6, 7), melatonin has also been demonstrated to be a potent antioxidant and neuroprotector against oxidative stress and β -amyloid toxicity (8–10). A recent study reported that melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic mouse model of AD (11). The decreased levels of melatonin in AD may thus be involved in the pathogenesis of AD. Indeed, recently we have found that melatonin levels in postmortem cerebrospinal fluid (CSF) decrease with the progression of AD neuropathology (as determined by the Braak stages) (12). Interestingly, CSF melatonin levels are already reduced in preclinical AD subjects that are cognitively still intact subjects and have only the earliest sign of AD neuropathology (Braak stages I-II) (12). These findings suggest that reduced melatonin levels may serve as an early

marker for the very first stages of AD that could so far not be monitored in any other way.

However, the mechanisms behind decreased melatonin levels in AD are not clear. Circulating melatonin levels are derived primarily from the pineal gland (13). Tryptophan (Trp) is taken up from the circulation and converted to serotonin (5-HT) by tryptophan hydroxylase (TPH). 5-HT is metabolized by the rate-limiting enzyme *N*-acetyltransferase (NAT) to *N*-acetyl-5-hydroxytryptamine, in turn by hydroxyindole-*O*-methyltransferase (HIOMT) into melatonin. Following its synthesis, pineal melatonin is passively secreted into the circulation. 5-HT can also be oxidized by monoamine oxidase A (MAOA) into 5-hydroxyindoleacetic acid (5-HIAA). Melatonin synthesis is influenced by light and regulated by the biological clock, *i.e.* the suprachiasmatic nucleus (SCN), through polysynaptic noradrenergic innervation, which involves binding of noradrenalin (NA) to the β_1 -adrenergic receptor on the pinealocytes to activate NAT. NA is mainly deactivated by MAOA into 3-methoxy-4-hydroxyphenylglycol (MHPG) (14–17) (Fig. 1A). In AD the neurons in the SCN become less active (18, 19), and noradrenergic fibers in the pineal seem to show dystrophic changes (20), which suggest the possibility of a disrupted noradrenergic regulation of the pineal in AD.

The present study aimed to clarify the molecular mechanisms underlying the decreased melatonin in preclinical AD and clinical AD. We systematically measured the precursors (tryptophan, 5-HT), products (melatonin, 5-HIAA), and

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; DA, dopamine; 5-HIAA, 5-hydroxyindoleacetic acid; HIOMT, hydroxyindole-*O*-methyltransferase; 5-HT, serotonin; HVA, homovanilic acid; MAO, monoamine oxidase; MHPG, 3-methoxy-4-hydroxyphenylglycol; NA, noradrenalin; NAT, *N*-acetyltransferase; SCN, suprachiasmatic nucleus; TPH, tryptophan hydroxylase; Trp, tryptophan.

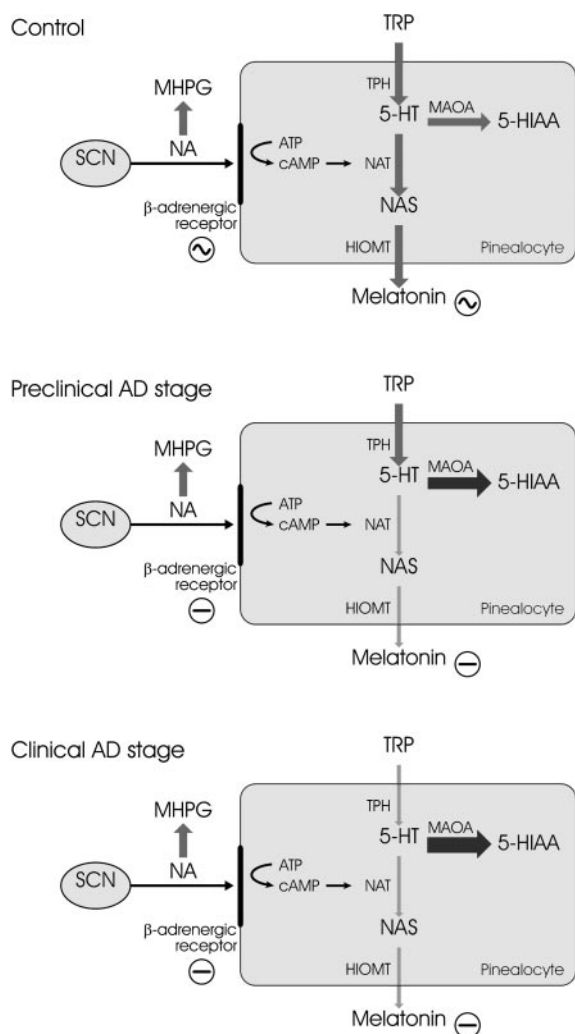


FIG. 1. A, Pineal melatonin synthesis pathway and its noradrenergic innervation in controls (Braak stage 0). B, In the preclinical AD subjects (Braak stages I–II), compared with controls, day/night rhythm of β_1 -adrenergic receptor mRNA disappears, which is responsible for the lack of melatonin diurnal rhythmicity. Nocturnal melatonin synthesis decreases, whereas the oxidation of its precursor 5-HT into 5-HIAA increases as indicated by the up-regulated MAOA. The metabolism of the noradrenergic regulatory system (*i.e.* MHPG:NA ratio) remains constant. C, In AD patients (Braak stage VI), compared with preclinical AD subjects, the conversion of Trp into 5-HT decreases, as indicated by the impaired TPH mRNA levels, which further contributes to the decrease of melatonin levels. ⊙ denotes circadian rhythmicity. ⊖ represents lack of circadian rhythmicity. Thicker arrows indicate up-regulated pathway. Thinner arrows denote down-regulated pathway.

mRNA levels of enzymes (TPH, NAT-1, HIOMT, MAOA, MAOB) in the melatonin synthesis as well as its noradrenergic regulation (NA, MHPG, β_1 -adrenergic receptor mRNA levels) of the pineal glands of controls (Braak stage 0), preclinical AD subjects (Braak stages I–II), and AD patients (Braak stage VI) (Fig. 1).

We conclude that the loss of melatonin diurnal rhythmicity and the decreased nocturnal melatonin levels, result from the dysfunction of noradrenergic regulation (*i.e.* β_1 -adrenergic receptor mRNA), and the depletion of its precursor 5-HT by

increased MAOA from the earliest preclinical AD stages onward.

Subjects and Methods

Subjects

Human brain material is obtained via the rapid autopsy system of The Netherlands Brain Bank (NBB), which supplies postmortem specimens from clinically well-documented and neuropathologically confirmed cases. Autopsies were performed according to the ethical code of conduct of the NBB on donors from whom written informed consent has been obtained either from the donor or direct next of kin. Permission was given for a brain autopsy and for the use of the brain tissue and clinical files for research purposes. Postmortem pineal glands were obtained at autopsy generally between 1 and 12 h after death and were immediately frozen in liquid nitrogen and kept at -80°C until assayed. Neuropathology of all subjects was systematically performed as previously described and the Braak staging was applied (21–23).

Pineal glands were studied from 76 subjects: 18 controls without any primary neurological or psychiatric disease and devoid of the AD neuropathologic changes (*i.e.* Braak stage 0), 33 cognitively intact cases with minor AD neuropathologic changes (*i.e.* Braak stages I and II), and 25 AD patients with extensive AD neuropathologic changes (*i.e.* Braak stage VI) (22). Subjects in Braak stages I–II have neurofibrillary changes in the transentorhinal region but did not show any clinical symptoms of AD (*i.e.* preclinical AD subjects) (22–24). Patients in Braak stages VI have severe neurofibrillary changes in neocortical area and clinically diagnosis of probable AD was performed according to the National Institute of Neurological and Communicative Disorders and Strokes–Alzheimer's Disease and Related Disorders Association criteria (25) with exclusion of other possible causes of dementia by history, physical examination, and laboratory tests (*i.e.* definite AD patients). Subjects who used β -adrenergic receptor blockers or antidepressants that might affect pineal melatonin levels (26–28) were not included.

The following variables were included in the present study: age, sex, clock time and date of death, postmortem delay, brain weight, pineal weight, and CSF-pH (*i.e.* a measure for agonal state) (29) (Table 1). To determine diurnal variations in pineal melatonin synthesis, according to the clock time of death the subjects were grouped into a day group (1000–2200 h) and a night group (2200–1000 h) because these periods are known to be associated with circadian differences in the levels of melatonin (30). In addition, to investigate the effect of photoperiods on pineal melatonin synthesis, according to the date of death, the subjects were grouped into a short photoperiod (September 23 to March 21) and a long photoperiod (March 21 to September 23) as described before (31). Age, sex, CSF-pH, day/night distribution, and short/long photoperiod distribution were well matched among the three groups (Table 1).

Sample preparation

Each frozen pineal gland was weighed and homogenized in liquid nitrogen. The powder from each pineal was divided into two parts. Part one of the homogenized pineal was weighed and suspended in 0.1 M perchloric acid (5 μl PCA per milligram powder), centrifuged at $12000 \times g$ for 15 min. Next, the supernatant was removed and used for total protein measurement (32), and melatonin, Trp, 5-HT, 5-HIAA, NA, MHPG, dopamine (DA), and homovanilic acid (HVA) assay. Part two of the homogenized pineal was used to measure TPH, NAT-1, HIOMT, MAOA, MAOB, and β_1 -adrenergic receptor gene expression using quantitative PCR (Fig. 1).

RIA

Melatonin levels were measured in the extracts of the postmortem pineal gland by a direct RIA (3, 33). The assay was run in a 0.1 M tricine buffer (Sigma, Zwijndrecht, The Netherlands) containing sodium chloride (0.15 M, Merck, Amsterdam, The Netherlands) and 0.1% gelatin (Merck) adjusted to pH 7.5. Iodinated melatonin (2-iodomelatonin, Amersham, Roosendaal, The Netherlands) was diluted in tricine buffer, at a final concentration of 25,000 cpm/ml. The melatonin antibody (AB/R/O3, Stockgrand, Guildford, UK) that was raised in rabbits is known to be highly specific. It cross-reacts with 6-hydroxymelatonin at 5.3% and

at less than 0.2% with 6-sulfatoxymelatonin (34). Standards were diluted in tricine buffer in a dilution range from 1 pg/ml to 1000 pg/ml. Each sample of the extract of the pineal gland was diluted 200 times with tricine buffer, and 200 μ l were pipetted in tubes and 200 μ l antimelatonin (final dilution 1:200,000) were added. The tubes were vortexed and incubated for 72 h at 4 C. Bound melatonin was separated by 50 μ l of donkey antirabbit antiserum coupled to cellulose (SAC-CEL, IOS, Boldon, UK). Precipitates were counted in a γ -counter (Cobra 500s, Packard, Groningen, The Netherlands). The intraassay coefficient was 8.7%.

HPLC

Fluorescence detection measurement. Tryptophan was measured in the extracts of the postmortem pineal gland by HPLC. The system consisted of a PU-1580 pump, an LG-1580-02 ternary gradient unit, a DG-980-50 3-line degasser, an AS-1555 autosampler, and a FP-920 fluorescence detector (Jasco, Maarsse, The Netherlands). Separation was performed on an XTerra C18 150 \times 4.6 mm (5 μ m) column with an XTerra guard column (Waters, Etten-Leur, The Netherlands). Tryptophan was eluted at 1.0 ml/min with a 0.005 M potassiumdihydrogen phosphate (pH 3) buffer solution and acetonitrile. All solid chemicals were from Sigma (Zwijndrecht, The Netherlands). Detection was performed at an excitation wavelength of 224 nm and an emission wavelength of 348 nm. Data were acquired and calculated with a Millennium32 (version 3.05) chromatographic data system (Waters). The accuracy and reproducibility of the method were both above 97%. Because of the technical limitation, we were not able to determine the NAS concentration in the supernatant with this system.

Electrochemical detection measurement. 5-HT, 5-HIAA, NA, MHPG, DA, and HVA were measured in the extracts of the postmortem pineal gland by HPLC as described before (35, 36). The chemicals used in this method were from Sigma. The mobile phase consisted of 10.4 mM citric acid, 6.1 mM sodium acetate, 1.60 mM heptanesulfonic acid, 0.4 mM EDTA, 11.8 mM sodium nitrate, and 12.5% methanol in water. It was pumped (Shimadzu LC-10ADvp, Den Bosch, The Netherlands) with a flow rate of 1.0 ml/min through a high-efficiency pulse dampener and reversed phase Supelcosil LC-18 DB, 250 \times 4.6 mm (Supelco, Zwijndrecht, The Netherlands), and similar guard column (25 \times 4.6 mm). Injector, pulse dampener, and columns were kept at 35 C in the oven compartment of an DECADE electrochemical detector workstation (ANTEC, Leiden, The Netherlands). The column was coupled to a Coulochem 5011 detector cell (Interscience, Breda, The Netherlands). The first electrode was operated at 300 mV, the second (measurement) electrode at –300 mV. Data were acquired and calculated with a Class-VP program (5.03 version,

Shimadzu). The accuracy and reproducibility were greater than 96% and greater than 97.5%, respectively.

RNA isolation and reverse transcription. Each frozen pineal gland was homogenized, and total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions (37). The RNA pellet was dissolved in diethylpyrocarbonate-treated distilled water and kept at –20 C. 1 μ g total RNA of each sample was denatured at 70 C for 10 min and annealed to 250 ng random hexanucleotides (Roche, Almere, The Netherlands), then reverse transcribed to cDNA by superscript II reverse transcriptase (Invitrogen) in 5 min at 30 C, 5 min at 37 C, and 90 min at 42 C in a final volume of 20 μ l, in the presence of Rnase inhibitor (Invitrogen). The cDNA was stored at –20 C until further use.

Quantitative PCR

Quantitative PCR was carried out in a final volume of 20 μ l in 96-well plates, using the SYBR Green PCR kit (Applied Biosystems, Foster City, CA) containing 2 μ l 10 \times SYBR Green PCR buffer, 1.6 μ l MgCl₂ (25 mM), 1.5 μ l dNTP blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP), 0.14 μ l AmpErase UNG (1 U/ μ l), 0.1 μ l Ampli Taq Gold (5 U/ μ l), 0.5 μ l cDNA sample (5 ng total RNA), and 3.0 μ l mixture of sense and antisense primers (each primer 2 pmol/ μ l). Cycling conditions were: 2 min at 50 C; 10 min at 95 C; 40 cycles of 15 sec at 95 C and 1 min at 60 C. The data were acquired and processed automatically by Sequence Detection Software (Applied Biosystems). The key enzyme of melatonin synthesis NAT has NAT-1 and NAT-2 subtypes (38, 39). Our preliminary data showed only NAT-1 transcripts in the human pineal gland, which is in accordance with *in situ* hybridization studies (40). MAO has two subtypes, MAOA and MAOB. MAOA is responsible for 5-HT and NA metabolisms (41). In the present study, we measured both MAOA and MAOB gene expression. Two reference genes were selected from a study of multiple adult and fetal tissues: EF-1- α and E2 ubiquitin conjugating enzyme (42) and were measured in all the samples to normalize expression data. The primers were designed with Primer Express software (Applied Biosystems). The efficiency of each primer pairs was calculated using cDNA dilution curves and linear regression. Details of the primers, the GenBank accession numbers, and the efficiency of each primer pair are given in Table 2. The mRNA expression levels of the two reference genes were highly correlated in our samples ($r = 0.913$; $P < 0.0001$) and were similarly expressed in the three groups ($P > 0.1$, $P > 0.17$, respectively). The amount of every target gene is calculated by raising the primer efficiency of the gene to the power of –cycle threshold, normalizing this and dividing by the average of the two normalized housekeeping gene expressions.

TABLE 1. Clinical and pathological data for the controls and AD patients studied (mean \pm SEM)

Group	Age (yr)	Sex (male/female)	Postmortem delay (h)	Day-night (day/night)	Photoperiod (long/short)	Brain weight (g)	Pineal weight (mg)	Pineal total protein content (mg/mg pineal)	CSF pH
Braak stage 0	68 \pm 2	18 (12/6)	6.8 \pm 0.3	11/7	7/11	1306 \pm 30	195.6 \pm 21	0.097 \pm 0.011	6.7 \pm 0.09
Braak stages I–II	73 \pm 1	33 (13/20)	7.7 \pm 0.5	14/19	15/18	1244 \pm 24	238.3 \pm 22	0.094 \pm 0.009	6.7 \pm 0.04
Braak stage VI	72 \pm 2	25 (10/15)	4.8 \pm 0.3	12/13	13/12	1106 \pm 27	244.8 \pm 25	0.118 \pm 0.015	6.6 \pm 0.06

According to the time of death, subjects were divided into “day group” (1000–2200 h) and “night group” (2200–1000 h). According to the date of death, subjects were grouped into “short photoperiod” (23 September–21 March) and “long photoperiod” (21 March–23 September).

TABLE 2. GenBank accession code, sequence of PCR primer pair for the target genes and reference genes, and amplification efficiency of each primer pair

Gene	Accession code	Forward primer	Reverse primer	Amplification efficiency
NAT-1	D90041	AGATGTGGCAGCCTCTGGAG	GCACCTGAGGCTGATCCTTC	1.90
HIOMT	U11090	CAGGTGGTGGCATTCTGGTA	CCTCGCTGTCTTCTATCCA	2.00
MAOA	M68840	TTCTGGCCTGCTGAAGATCAT	CCTAGGGCAGTTACTGATGTG	1.95
MAOB	M69177	TTTTCAGCAACGGCTCTTGG	CACAAGTAGCCCCCTTTTGTG	2.08
β_1 -adrenergic receptor	NM_000684	CCCACAATCCTCGTCTGAATC	AGGAACATCAGCAAGCCACTC	1.94
TPH	X52836	CTCTTAGGTCATGTCCCGCTTT	GGAGAATTGGGCAAACTAGGTT	1.90
EF-1- α	J04617	AAGCTGGAAGATGGCCCTAAA	AAGCGACCCAAAGGTGGAT	1.95
E2 ubiquitin	U39317	CTGAAGAGAATCCACAAGGAATTGA	CTCCAACAGGACCTGTGTAAC	1.94

Statistics

Differences of monoamine levels and target gene expression levels among the three groups were tested by the Kruskal-Wallis test. Differences between groups were tested using the Mann-Whitney *U* test. The differences in proportion between males and females, the number of subjects that died during the day and night, and the number of subjects that died during the short photoperiod and long photoperiod for the three groups were tested by χ^2 . Correlations were analyzed by the Spearman correlation test. Differences were considered statistically significant at the $P < 0.05$ level (two-tailed).

Results

No significant difference was found in pineal weight and pineal total protein content between the three sample groups (Braak stage 0, stages I-II and stage VI) ($P = 0.62$, $P = 0.215$) (Table 1). No significant correlation was observed between postmortem delay, brain weight on the one hand, and all the monoamine levels and gene expression levels measured on the other.

Melatonin levels in the CSF closely reflect the pineal melatonin content

We compared the data from the 41 subjects whose melatonin levels were measured in both the CSF (12) and pineal gland (present study). A highly positive correlation between melatonin levels in the CSF and pineal was found ($r = 0.83$, $P < 0.0001$, $n = 41$) (Fig. 2), indicating that the CSF alterations in melatonin levels reflected the changes in the pineal melatonin content.

Changes in the pineal melatonin synthesis (Fig. 1)

The day/night differences of melatonin and melatonin/5-HT ratio (*i.e.* melatonin synthesis activity) found in Braak stage 0 ($P = 0.012$, $P = 0.001$, respectively) had disappeared in Braak stages I-II and Braak stage VI (Fig. 3, A and B). No day/night difference of other monoamines or mRNA levels of the enzymes involved in melatonin synthesis was found in the three groups or any photoperiodic difference in monoamines or enzymes mRNA levels in melatonin synthesis.

Nocturnal melatonin levels decreased in Braak stages I-II

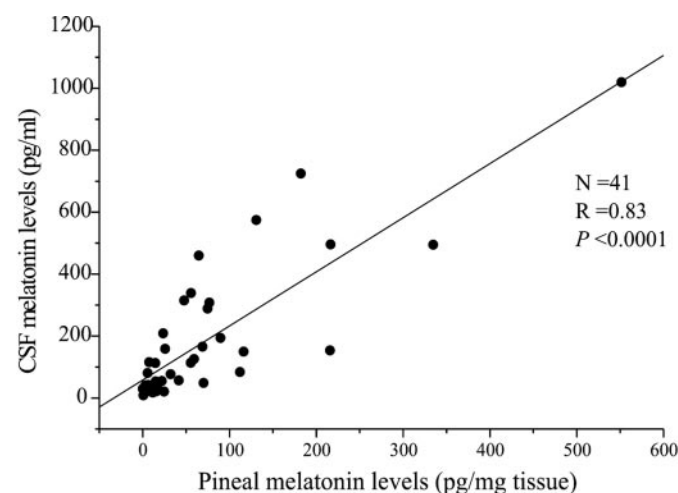


FIG. 2. The significant positive correlation between melatonin levels in the CSF and pineal gland.

and Braak stage VI (Table 3 and Fig. 3A). Nocturnal melatonin/5-HT ratio (*i.e.* melatonin synthesis activity) decreased in Braak stage VI, compared with Braak stage 0 ($P = 0.012$) (Table 3 and Fig. 3B). NAT-1 mRNA levels showed a trend to increase in Braak stage VI ($P = 0.054$) (Table 3 and Fig. 3C), whereas HIOMT mRNA levels seemed to decrease in Braak stage VI ($P = 0.068$) (Table 3).

5-HIAA levels, the oxidative product of 5-HT by MAOA, were higher in Braak stage VI, compared with Braak stage 0 ($P = 0.003$) and Braak stages I-II ($P = 0.018$) (Table 3 and Fig. 3D). Moreover, the MAOA mRNA levels and 5-HIAA/5-HT ratio (*i.e.* MAOA activity) were step-wise increased in Braak stages I-II ($P = 0.037$, $P = 0.040$, respectively) and Braak stage VI ($P < 0.0001$, $P = 0.007$, respectively) compared with Braak stage 0 (Table 3 and Fig. 3, E and F). The 5-HIAA:5-HT ratio (*i.e.* MAOA activity) and MAOA mRNA levels correlated positively ($r = 0.370$, $P = 0.001$, $n = 76$). In addition, MAOB mRNA levels were increased in Braak stage VI, compared with Braak stage 0 ($P = 0.024$) and Braak stage I-II ($P = 0.015$) (Table 3). No correlation between the 5-HIAA:5-HT ratio and MAOB mRNA levels was found in the three groups.

The concentration of Trp, the precursor of 5-HT and melatonin, was higher in Braak stage VI, compared with Braak stage 0 ($P = 0.025$) and Braak stages I-II ($P = 0.004$) (Table 3 and Fig. 3G). The mRNA levels of TPH, the key enzyme for the conversion of Trp to 5-HT, were lower in Braak stage VI, compared with Braak stage 0 ($P = 0.022$) and Braak stages I-II ($P = 0.027$) (Table 3 and Fig. 3H). No significant difference of 5-HT or the 5-HT:Trp ratio was found among the three groups ($P = 0.193$, $P = 0.326$, respectively) (Table 3).

Dysregulated noradrenergic system (Fig. 1)

A day/night difference of β_1 -adrenergic receptor mRNA levels was present in Braak stage 0 ($P < 0.001$) but disappeared in Braak stages I-II and Braak stage VI ($P = 0.135$, $P = 0.174$, respectively) (Fig. 4). There were no day/night differences in NA or MHPG concentrations in the three groups. No photoperiodic difference in either of these factors was found in the three groups.

MHPG concentration in Braak stage VI was higher than Braak stage I-II ($P = 0.001$) and insignificantly higher than Braak stage 0 ($P = 0.09$). No significant difference of NA, MHPG:NA ratio (*i.e.* NA metabolic activity) and β_1 -adrenergic receptor mRNA levels ($P = 0.47$, $P = 0.26$, $P = 0.437$, respectively) was found among the three groups (Table 3 and Fig. 5).

β_1 -Adrenergic receptor mRNA levels were correlated with melatonin levels ($r = 0.45$, $P < 0.0001$, $n = 76$) and melatonin/5-HT (*i.e.* melatonin synthesis activity) ($r = 0.37$, $P < 0.001$, $n = 76$).

Dopaminergic system

No day/night or photoperiodic differences in either of DA and HVA concentrations or HVA:DA ratio (*i.e.* DA metabolic activity) were present in the three groups. No significant differences of these parameters were found in the three groups, indicating that the dopaminergic system is not involved in the changes of melatonin in AD (Table 3).

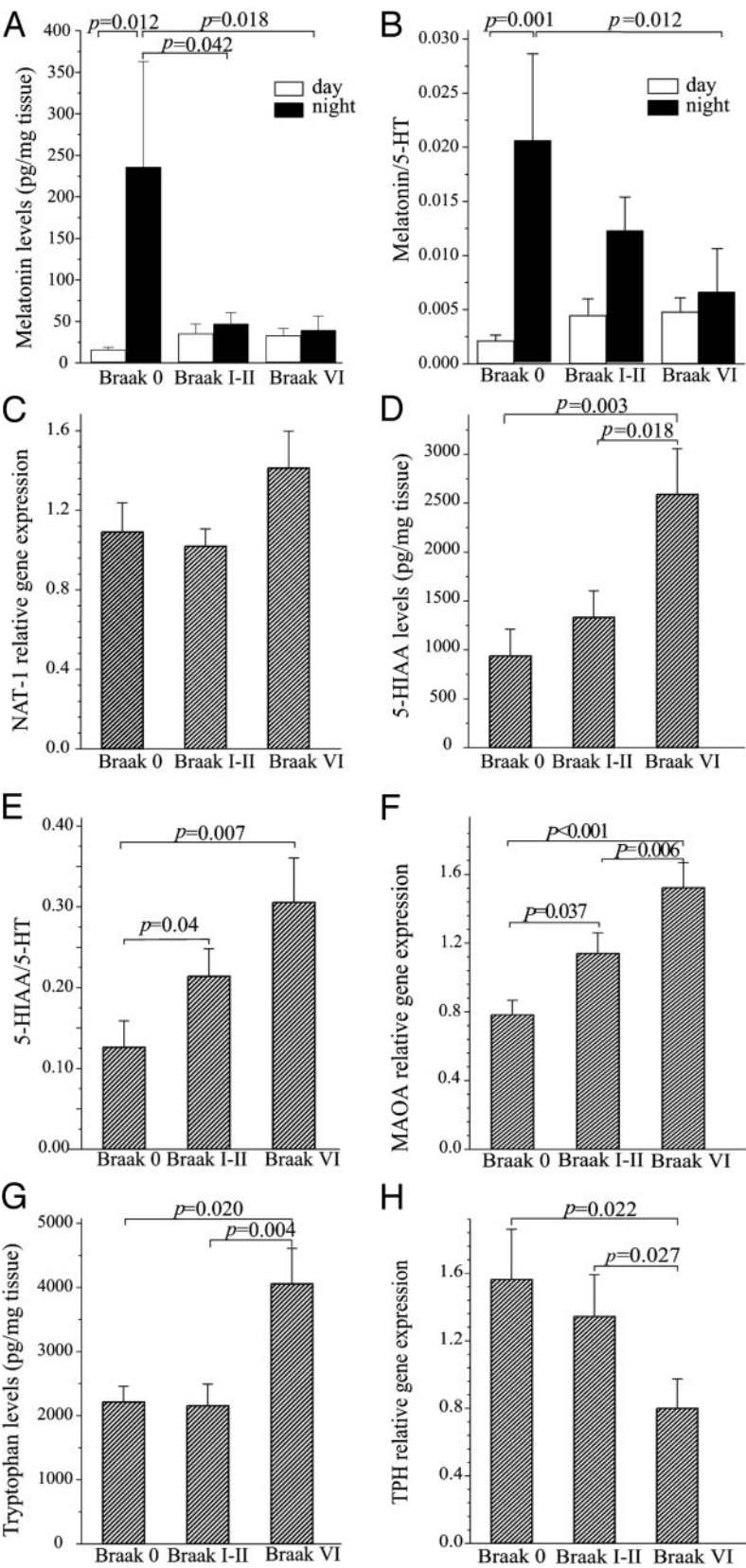


FIG. 3. A, Day/night difference of melatonin is present in Braak stage 0 but disappears in Braak stages I-II and stage VI. Nocturnal melatonin levels are decreased in Braak stages I-II and stage VI. B, Day/night difference of melatonin/5-HT (representing the melatonin synthesis activity) is present in Braak stage 0 but is lost in Braak stages I-II and stage VI. Melatonin/5-HT decreases in Braak stage VI, compared with Braak stage 0. C, NAT-1 gene expression tends to be increased in Braak stage VI (Kruskal-Wallis test, $P = 0.054$). D, The levels of 5-HIAA, the oxidative product of 5-HT, are elevated in Braak stage VI. E, 5-HIAA/5-HT, representing the activity of MAOA, is increased in Braak stages I-II and stage VI. F, MAOA gene expression is stepwise increased in Braak stages I-II and stage VI. G, Tryptophan levels are increased in Braak stage VI. H, The gene expression of TPH is reduced in Braak stage VI.

TABLE 3. Mean concentrations of monoamines and mean target gene relative expression levels in the pineal glands of Braak stages I–II and Braak stage VI groups, and the percentages of Braak stage 0 group (controls)

	Braak stages I–II		Braak stage VI		Difference between Braak stages I–II and VI
	Mean \pm SEM	%	Mean \pm SEM	%	
Trp	2996 \pm 531	87	5890 \pm 844	170 ^a	<i>b</i>
5-HT	7348 \pm 1131	82	9152 \pm 1204	102	
Melatonin ^c	46.0 \pm 13.2	20 ^a	35.3 \pm 16.8	15 ^a	
5-HIAA	1336 \pm 267	142	2593 \pm 466	276 ^b	<i>a</i>
5-HT/Trp	4.5 \pm 1.0	109	2.4 \pm 0.5	60	
Melatonin/5-HT	0.012 \pm 0.003	59	0.007 \pm 0.004	32 ^a	
5-HIAA/5-HT	0.21 \pm 0.03	162 ^a	0.31 \pm 0.05	238 ^b	
NA	61.4 \pm 8.9	124	63.9 \pm 7.6	129	
MHPG	112.5 \pm 13.8	88	179.0 \pm 29.1	139	<i>b</i>
MHPG/NA	4.2 \pm 0.9	100	3.6 \pm 0.6	86	
DA	16.2 \pm 5.5	107	12 \pm 1.7	79	
HVA	185.8 \pm 16.6	110	198.3 \pm 25	116	
HVA/DA	31.3 \pm 7.0	136	22.4 \pm 3.8	96	
TPH	1.35 \pm 0.25	86	0.80 \pm 0.17	51 ^a	<i>a</i>
NAT-1	1.02 \pm 0.09	94	1.41 \pm 0.18	129	
MAOA	1.14 \pm 0.11	146 ^a	1.52 \pm 0.72	195 ^b	<i>b</i>
MAOB	0.93 \pm 0.15	109	1.41 \pm 0.19	166 ^a	<i>a</i>
HIOMT	0.98 \pm 0.10	77	0.76 \pm 0.07	60	
β_1 -adrenergic receptor	1.06 \pm 0.10	108	1.13 \pm 0.10	122	

Mean values of monoamines are expressed as picograms per milliliter (mean \pm SEM). Genes relative expression data are expressed as normalized data (mean \pm SEM).

%, Percentage of the respective mean values of the control group (Braak stage 0).

^a $P < 0.05$; ^b $P < 0.01$.

^c Nighttime value.

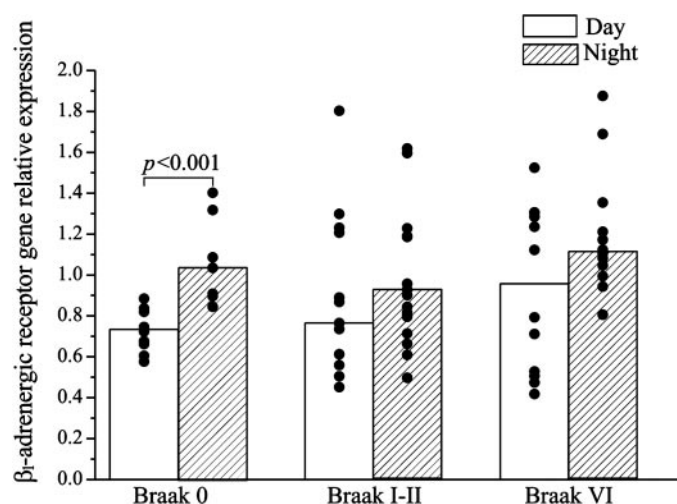


FIG. 4. Day/night rhythm of β_1 -adrenergic receptor gene expression in Braak stage 0 disappears in Braak stages I–II and stage VI. Bar indicates the median of β_1 -adrenergic receptor gene expression. Each single point indicates the β_1 -adrenergic receptor gene expression of one subject. Note the considerable variations within the groups, especially in Braak stages I–II and stage VI.

Discussion

We have taken a comprehensive approach to study changes in the melatonin synthesis pathway during AD. As could be expected, this approach for instance yields a significant positive correlation between the mRNA level of MAOA and the ratio of the substrate and product of MAOA, 5-HT, and 5-HIAA. Moreover, we observed positive correlations between steps in the metabolic pathway that are much more distant such as between the mRNA level of the β_1 -adrenergic receptor and melatonin. This suggests that

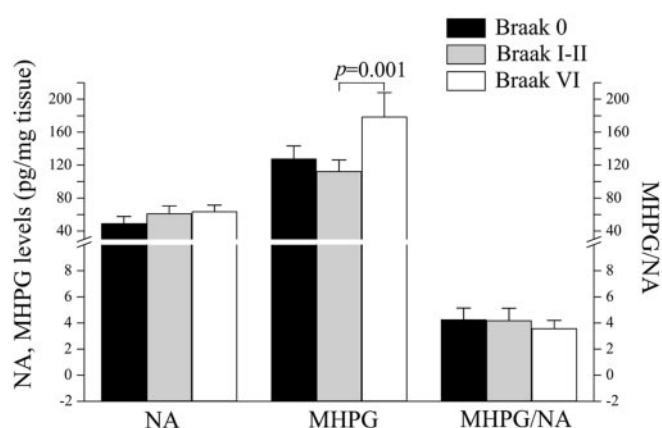


FIG. 5. MHPG levels in Braak stage VI are higher than in Braak stage I–II ($P = 0.001$) and insignificantly higher than in Braak stage 0 ($P = 0.09$). No difference in NA levels and MHPG:NA ratio (reflecting the metabolic activity of noradrenergic system) between Braak stage 0, stages I–II, and stage VI.

melatonin synthesis is mainly regulated by noradrenergic regulation in humans and justifies our approach. More novel observations were made in our study. In both Braak stages I–II and Braak stage VI, there was a shift in melatonin synthesis pathway: an increased oxidation of 5-HT to 5-HIAA with up-regulated MAOA and an impaired conversion of 5-HT to melatonin, in addition to the dysregulated β_1 -adrenergic receptor mRNA, which are responsible for the decreased nocturnal melatonin synthesis and the loss of melatonin diurnal rhythm in the preclinical AD subjects and AD patients (Fig. 1, B and C). The decreased TPH mRNA levels in Braak stage VI may further decrease melatonin synthesis in AD patients (Fig. 1C). Also, the observed highly positive correlation between melatonin levels in the pineal and in the

CSF indicates that the decreased CSF melatonin levels in AD are due to the reduced pineal melatonin synthesis rather than the dilution of the CSF in AD (43) (Fig. 2).

The diurnal rhythm of pineal melatonin disappeared and the nocturnal melatonin levels decreased in Braak stages I-II and Braak stage VI (Fig. 3A), which is in full agreement with the reduced melatonin levels in preclinical AD subjects (12) and AD patients (5, 44). Moreover, these findings may explain why earlier studies (3, 4) did not find melatonin circadian rhythm in aged controls, which were not Braak staged and will thus have been combinations of Braak stage 0 controls and preclinical AD subjects. Although the latter group did not show the clinical symptoms of AD, the diurnal melatonin rhythm was already strongly diminished (Fig. 3A).

The day/night rhythm of melatonin synthetic activity (*i.e.* melatonin/5-HT) found in Braak stage 0 had disappeared in Braak stages I-II and Braak stage VI (Fig. 3B). However, neither AD-related changes nor day/night rhythms of NAT-1 mRNA or HIOMT mRNA were found in the present study. These observations are supported by studies in the rhesus macaque (45), which showed that NAT activity plays a crucial role in the melatonin rhythmicity, whereas NAT activity is not regulated by changes in NAT mRNA (46) but rather by the posttranscriptional control, *e.g.* proteasomal proteolysis (47). Whether the posttranscriptional regulation of NAT is affected in the progression of AD remains to be clarified.

In contrast to the decreased melatonin production from 5-HT, the oxidation of 5-HT to 5-HIAA was strongly and stepwise increased, as indicated by the elevated MAOA activity (*i.e.* 5-HIAA:5-HT ratio) and mRNA levels in Braak stages I-II and Braak stage VI (Figs. 1, B and C, and 3, E and F). Increased MAOA activity and mRNA levels as found in the pineal gland in the present study seem to be a general phenomenon in AD because it was also reported in the cortex, thalamus, hypothalamus, and white matter of AD patients (48–50). Interestingly, MAOA gene polymorphisms are suggested to be associated with an increased susceptibility for AD (51). The elevated 5-HIAA levels found in the pineal of AD patients differ from the decreased 5-HIAA levels in cortex, amygdala, and caudate nucleus of AD patients (52, 53). This difference may be related to the fact that the predominant distribution of 5-HT in the pineal is cytosolic, whereas 5-HT is stored in subcellular vesicles in the midbrain system (54). Therefore, in the pineal, 5-HT may be more vulnerable to the oxidation by MAOA to 5-HIAA than in the rest of the brain. Our data suggest that reduced melatonin production in preclinical AD subjects and AD patients may be due to the depletion of its precursors 5-HT, caused by the up-regulation of MAOA. In fact, MAOA inhibitors significantly increase serum melatonin levels in human and rodents (27, 28, 55, 56).

In the present study, a day/night rhythm of β_1 -adrenergic receptor mRNA with elevated levels at night, present in Braak stage 0, was absent in Braak stages I-II and Braak stage VI (Fig. 5). This finding suggests that the dysregulation of β_1 -adrenergic receptor mRNA is the basis of the lack of day/night rhythms of melatonin we observed in Braak stages I-II and Braak stage VI. It has been shown in rats that the levels of pineal β -adrenergic receptor mRNA are de-

creased, and its diurnal rhythm is abolished on removal of the sympathetic innervation from the SCN (57, 58). Our earlier observations revealed a marked decrease of vasopressin expression neuron numbers, activity, and circadian rhythmicity in the SCN of AD patients (18, 19, 59). Taking these data together, we hypothesize that the circadian fluctuations of the SCN are affected already in the earliest preclinical stages of AD, which results in a dysregulation of β_1 -adrenergic receptor mRNA and thus in a decrease of nocturnal melatonin synthesis and the disappearance of the diurnal melatonin rhythm. Studies on the circadian rhythm of the SCN in the first Braak stages should be performed to confirm this idea.

No day/night rhythm of NA, MHPG, or MHPG:NA in the pineal gland was found in Braak stage 0, stages I-II, or stage VI. This may well be explained by the fact that NA and MHPG levels in the homogenized pineal we measured are the dilution of the levels in the noradrenergic terminal, which directly reflect the day/night stimulus from the SCN. MHPG concentration was increased in AD patients probably because of the increased MAOA, whereas the metabolism of the NA system remained constant, as indicated by the constant MHPG:NA ratio in Braak stages I-II or stage VI (Table 3 and Fig. 4).

Although the groups did not show a difference in possible confounding factors such as age, gender, or CSF pH (a measure for agonal state) (29, 60), a limitation of the present postmortem study is that it is not known whether the clinical condition of the patients in the various groups, including the circumstance and the time of death, may have influenced the results.

In summary, the dysregulation of pineal β_1 -adrenergic receptor mRNA and the increased MAOA activity and mRNA levels are held responsible for the disappearance of the melatonin diurnal rhythm and the decrease of nocturnal melatonin synthesis in preclinical AD subjects and AD patients. In addition, the decreased TPH mRNA levels may further contribute to this change in AD patients (Fig. 1). Our finding of a lack of circadian β_1 -adrenergic receptor mRNA rhythm in Braak stages I-II suggests that the first alterations may take place in the SCN in the earliest preclinical stages of AD pathology.

These findings support the possibility of reduced melatonin levels as an early marker for the onset of AD and provide a basis for a mechanism behind bright light therapy to restore circadian rhythm disorders in AD (61). Furthermore, because the loss of melatonin rhythmicity already occurs in people with early AD neuropathology, before clinical symptoms occurs, it may be beneficial to supplement melatonin in case of decreased nocturnal melatonin levels to slow down the development of AD. The recent finding that melatonin increases survival and inhibits amyloid pathology in an Alzheimer mouse model (11) supports this possibility. Whether the increased MAOA, a general phenomenon in the AD brain (48–50), contributes to the symptoms of AD, *e.g.* to depression or to the pathogenesis of AD, demands further investigation. In that case, therapeutic use of MAOA inhibitors in AD may be considered.

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