Decreased Vasopressin Gene Expression in the Biological Clock of Alzheimer Disease Patients With and Without Depression

RONG-YU LIU, MD, JIANG-NING ZHOU, MD, PhD, WITTE J.G. HOOGENDIJK, MD, PhD, JOOP VAN HEERIJHUIZE, WOUTER KAMPORST, MD, UNGA A. UMMEHOPA, MICHEL A. HOFMAN, PhD, AND FICK D. SWAAB, MD, PhD

Abstract. Circadian rhythm disturbances are frequently present in Alzheimer disease (AD). In the present study, we investigated the expression of vasopressin (AVP) mRNA in the human suprachiasmatic nucleus (SCN). The in situ hybridization procedure on formalin-fixed paraffin-embedded material was improved to such a degree that we could, for the first time, visualize AVP mRNA expressing neurons in the human SCN and carry out quantitative measurements. The total amount of AVP mRNA expressed as masked silver grains in the SCN was 3 times lower in AD patients (n = 14; 2,135 ± 597 μm²) than in age- and time-of-death-matched controls (n = 11; 6,667 ± 1466 μm²) (p = 0.003). No significant difference was found in the amount of AVP mRNA between AD patients with depression (n = 7) and without depression (n = 7) (2,985 ± 1103 μm² and 1,285 ± 298 μm², respectively; p = 0.38). In addition, the human SCN AVP mRNA expressing neurons showed a marked day-night difference in controls under 80 years of age. The amount of AVP mRNA was more than 3 times higher during the daytime (9,028 ± 1709 μm², n = 7) than at night (2,536 ± 740 μm², n = 4; p = 0.02), whereas no clear diurnal rhythm of AVP mRNA in the SCN was observed in AD patients. There was no relationship between the amount of AVP mRNA in the SCN and age at onset of dementia, duration of AD and the neuropathological changes in the cerebral cortex. These findings suggest that the neurobiological basis of the circadian rhythm disturbances that are responsible for behavioral rhythm disorders is located in the SCN. It also explains the beneficial effects of light therapy on nightly restlessness in AD patients.

Key Words: Circadian rhythms; Aging; Alzheimer disease; Human Brain; Suprachiasmatic nucleus; Vasopressin.

INTRODUCTION

Sleep disruption, nightly restlessness, sundowning, and other circadian rhythm disturbances are frequently seen in Alzheimer disease (AD) patients (1–4). In fact, nocturnal restlessness is the main cause of hospitalization of these patients (5). The neurobiological basis of these behavioral disorders is thought to be a degenerative change in the suprachiasmatic nucleus (SCN), the clock of the brain, which generates and coordinates circadian rhythms, such as the sleep-wake cycle, and hormonal rhythms (6–7). Furthermore, it has been hypothesized that circadian rhythm disturbances may contribute to mental decline and depressive mood in AD (8). Exposure of aged rats to bright light appeared not only to reverse age-related alterations in circadian sleep-wake rhythm disturbance (9), but also to prevent the age-related decrease in the number of vasopressin (AVP) immunoreactive neurons in the SCN (10). In AD the number of AVP immunoreactive neurons in the SCN is strongly diminished (11–12) and following exposure to extra amounts of bright light, circadian rhythm disorders appeared to be improved in AD (3, 13). These findings support the idea that degenerative changes in the SCN may be the biological basis of circadian disturbances in aging and AD, and that they can be reversed by stimulation of the SCN by light. The aim of the present study was to establish whether the degenerative changes in the SCN of AD patients are indeed accompanied by decreased AVP gene expression of the clock. In addition, we determined whether there was a relationship between the amount of AVP mRNA in the SCN and the presence or absence of depression in AD patients.

MATERIALS AND METHODS

Subjects

Demented patients were studied in the framework of a longitudinal study of depressive symptoms in AD patients in 8 nursing homes. After their death, brain autopsy was performed on patients and controls as part of the program of the Netherlands Brain Bank. Written informed consent for brain autopsy, the use of the tissue, and medical records for research purposes was obtained before subjects entered the study. Brains of 14 AD patients were collected and matched with 11 controls for age, sex, and clock time of death (Table 1). The diagnosis of AD and control subjects was neuropathologically confirmed (Dr. W. Kamphorst, Free University, Amsterdam). On the basis of the psychiatric evaluation, the AD patients were divided into a group with a major depressive episode (n = 7), and a group without depression (n = 7). In order to match for the diurnal variations in the AVP neuron population, subjects were grouped...
into 2 periods based on the clock time of death: 10:00 AM to 10:00 PM, and 10:00 PM to 10:00 AM, since these periods are known to be associated with differences in the degree of physiological and behavioral circadian rhythmicity, as well as in the number of SCN neurons expressing AVP (14–16).

### Clinical Evaluation

Possible and probable AD was diagnosed according to the NINCDS-ADRDA (17) and DSM-III-R criteria (18). Severity of clinical dementia in the early stages of AD was determined by the Mini Mental State Examination (MMSE) (19). At 6-month intervals, the Global Deterioration Scale (GDS) (20) and Functional Assessment Staging (FAST) (21) were performed. Immediately postmortem, 1 additional GDS was scored based upon observations on the severity of dementia in the last 2 weeks of life. Mean interval between the last clinical measurement and death was 3.3 ± 0.7 months. DSM-III-R criteria were used to diagnose a major depressive episode. The severity of the depressive symptoms was determined at 6-month intervals by the Hamilton Depression Rating Scale (22) and the Cornell Scale for the Assessment of Depression in Dementia (23). Patients in the depressed AD group suffered from a major depressive episode according to DSM-III-R at death, and had been severely depressed for at least 3 months. All nondepressed AD patients were free of mood disorders throughout the AD process, and did not have a history of depression. Furthermore, depressed and nondepressed AD patients were matched for the severity of clinical dementia, as measured by the last FAST before death (ranging from stage 6c to 7f) and by the GDS at death (p = 0.7). MMSE scores during the last measurement were too low to be of discriminative value. The 2 AD groups were also comparable as far as the type of psychiatric comorbidity and medication used were concerned (Table 2).

### Neuropathological Evaluation

After a standard fixation time of 4 weeks, a neuropathologist (WK) confirmed the diagnosis of AD on the basis of the presence of many neuritic plaques, neurofibrillary tangles, neurit threads, and dystrophic neurites in Bodian- and Congo red-stained sections from the hippocampus and cortex (frontal area 10 or 11; temporal area 22 or the temporal pole; parietal area 7 or 40; occipital area 17 and 18). To exclude Parkinson changes, the substantia nigra was also examined (24). All AD patients fulfilled the CERAD criteria for AD (25). In addition, the neuropathologist (WK) performed a semiquantitative estimation of the severity of AD changes according to the classification of Braak (26). On the basis of the location of the AD pathology, a score of 0–VI was assigned to the patients (26). The AD

### Table 1

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<th>PMD (hours)</th>
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AD: Alzheimer disease; BW: Brain weight; PMD: postmortem decay; f: female; m: male; NBB: Netherlands Brain Bank number.
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<th>Medication (last 3 months)</th>
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<td>Mean ± SEM</td>
<td>75 ± 4</td>
<td>9 ± 1</td>
<td>6 ± 1**</td>
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<td>5.00 ± 0.30</td>
<td>1,285 ± 298</td>
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AD: Alzheimer disease. FAST: Functional Assessment Staging; NBB: Netherlands Brain Bank; SCN: suprachiasmatic nucleus. **: p < 0.0004. *: According to Braak; #: number of profiles expressed AVP mRNA in the SCN.
patients had a score of V–VI and the controls had a score of 0–II. Moreover, a more differentiated semiquantitative sum score of neurofibrillary tangles, neuritic plaques, and disruption of the neuropil was performed in a Bodian staining of the frontal, temporal, parietal, and occipital cortex. In brief, in each area all AD changes were separately scored as 0 = absent, 1 = present but less than moderate, 2 = moderate (i.e. 2 to 3 neurofibrillary tangles, 2 to 3 neuritic plaques, or 30%–60% of the normal network replaced by neuropil threads per 0.4 mm² area), and 3 = more than moderate (27–28). The sum scores of the controls were 3 ± 0.9 (mean ± SEM), and those of the Alzheimer patients were 25 ± 2 and did not overlap.

Measurement of AVP mRNA in SCN

In situ hybridization was performed on every fifteenth 6-μm section of the SCN. The AVP probe (hvp3) consisted of an oligomer of 48 nucleotides complementary to bases 411–458 of the preprovasopressin precursor (29). The specificity of the probes has been described previously (30). The probe was 3’-end labeled using terminal deoxynucleotidyltransferase (Boehringer Mannheim) and [α-35S] dATP, as described earlier. Tissue pretreatments were performed mainly as previously described (31) except for some improvements in the deproteination and delipidation steps. Deproteinization was performed in Proteinase-K for 15 min instead of 30 min. Delipidation was done in 0.1% Triton X-100 in PBS for 10 min and sections were washed in PBS without dehydration before hybridization. These steps improved the signal to noise ratio. Each section was incubated with 68 μl hybridization solution containing an approximate 1 × 106 cpm labeled probe. After overnight incubation at 42°C, the sections were rinsed in 1 × SSC for 30 min at 50°C, 2 × 30 min 0.1 × SSC at 50°C, and 2 × 30 min 0.1 × SSC at room temperature. Sections were dehydrated at room temperature in 300-mM ammonium acetate (pH 5.5)/ethanol 100% at volume ratios 1:1, 3:7, 1:9, and 0:1. In order to check the autoradiographic signal, sections were exposed to β-max hyperfilm (Amersham, UK) for 2 days. Subsequently, slides were dipped in photographic emulsion (NTB2 Kodak USA) and exposed for 17 days. Slides were developed for 2 min in Dektol Developer (Sigma) at 15°C, briefly rinsed in aquadest at 15°C, and fixed in Kodak fixer (Sigma) for 14 min. Sections were washed to remove the fixative and counter-stained with thionin.

Quantitative Analysis of AVP mRNA

For quantitative analysis of the in situ signal of the AVP mRNA in the SCN, an IBAS-KAT image analysis system was connected to a Bosch TYK9B TV camera equipped with a chalycon tube mounted on a Zeiss microscope. The microscope was equipped with planapo objectives and a scanning stage. The microscope was connected to a Bosch TYK9B TV camera equipped with a chalycon tube mounted on a Zeiss microscope. The microscope was equipped with planapo objects and a scanning stage. The microscope was connected to a Bosch TYK9B TV camera equipped with a chalycon tube mounted on a Zeiss microscope.

Statistical Analysis

Differences in amount of AVP mRNA in the SCN or total number of AVP mRNA expressing cell profiles between the groups were tested using the Mann-Whitney U test. To determine the effects of both clock time and age on the amount of AVP mRNA in the SCN morphology, data were analyzed using a 2-factor analysis of variance (ANOVA). Correlation of brain weight, postmortem interval, pathological changes, Cornell scores, and impairment of cognitive function versus amount of AVP mRNA were analyzed by Spearman correlation coefficients in AD patients. A significance level of 5% was used in all statistical tests (2-tailed). Throughout this paper, values are expressed as mean ± standard error of the mean (SEM).

RESULTS

Decreased AVP mRNA in AD

The in situ hybridization procedure on formalin-fixed paraffin-embedded material was improved to such a degree that we could show and quantify, for the first time, AVP mRNA expressing cell profiles in the human SCN (Fig. 2A). A clearly decreased amount of AVP mRNA was found in the SCN of AD patients (Fig. 2B, C). The total masked area of silver grains in cells, being an estimate of total amount of AVP mRNA in the SCN, was reduced by 68% in AD patients (2,135 ± 597 μm²) as compared with controls (6,667 ± 1,466 μm²) (p = 0.003). There was also a 60% decrease in the total number of profiles that expressed AVP mRNA in AD compared with those in controls (7,233 ± 7,227 and 23,227 ± 3,471 respectively, p = 0.0005). No significant difference was found in the amount of AVP mRNA between AD patients with depression (n = 7) and without depression (n = 7) (2,985 ± 1,103 μm² and 1,285 ± 298 μm², respectively; p = 0.38), or in the total number of AVP mRNA expressing profiles (11,360 ± 2,764 and 7,906 ± 1,616, respectively; p = 0.46). Therefore the AD patients with and without depression were put into one group for further analysis.

Diurnal Variations

Two-way analysis of variance revealed that age and time of death had an interaction effect on the amount of AVP mRNA in the SCN of control subjects (p = 0.004). The human SCN AVP mRNA expressing neurons showed a marked day-night difference in controls (Fig.
Fig. 1. Quantitative analysis of the in situ signal of AVP mRNA in the SCN. A: The area of the SCN was manually outlined at low magnification and a grid of fields was superimposed. From this grid 50% of the fields indicated by red rectangles were randomly selected and stored. III: third ventricle. OC: optic chiasm. SON: supraoptic nucleus. B: At high magnification each field was retrieved at high resolution on the image analysis monitor.

3). The amount of AVP mRNA expressed as masked silver grains was more than 3 times higher during the daytime (9,028 ± 1,709 μm², n = 7) than at night (2,536 ± 740 μm², n = 4; p = 0.02). However, such a day-night difference in SCN AVP mRNA was only found in control subjects younger than 80 years. There were no obvious daily fluctuations in AVP mRNA in 4 controls over 80 years of age. In controls who died during the day period (10:00 AM to 10:00 PM), the amount of AVP mRNA was 4 times higher (9,028 ± 1,709 μm², n = 7) than in AD patients (1,971 ± 773 μm², n = 10; p = 0.001). There was a significant difference in the total number of profiles expressing AVP mRNA between controls and AD patients (27,937 ± 4,536, n = 7; 8,538 ± 1,569, n = 10 respectively; p = 0.0004) during daytime. In the SCN, no such difference was observed between controls and AD patients during the night period.

Decreased AVP mRNA During Aging

In controls, a significant decrease in the amount of AVP mRNA was found with aging. The total number of profiles that expressed AVP mRNA in the SCN in subjects over 80 years of age (n = 4, 14,020 ± 1,400) was considerably lower than those in 60- to 80-year-old controls (n = 7, 28,488 ± 4,278, p = 0.03). Controls over 80 years of age (n = 4) generally had an amount of AVP mRNA (2,859 ± 712 μm²) which was 3 times lower than that in controls from 60 to 80 years of age (n = 7, 8,844 ± 1,821 μm²). However, this difference failed to reach statistical significance (p = 0.16), apparently due to the

Fig. 2. Thionin-counterstained emulsion autoradiograms in frontal sections (6 μm) of the human suprachiasmatic nucleus (SCN) at high magnification. Note the black silver deposits that show the presence of vasopressin (AVP) mRNA in SCN neurons. There are also thionin-stained AVP mRNA negative cells present in the SCN. A: Control subject. B: AD patients with depression. C: AD without depression. Note there were less AVP mRNA expressing neurons in AD patients, while no difference was seen between the AD patients with and without depression. Scale bar = 100 μm.
Fig. 3. Day-night fluctuation in AVP mRNA of the SCN (expressed as masked area of silver grains) in controls and AD patients. Note that at any moment of the day, the values for AD patients are lower than those for controls. ▲: Controls younger than 80 years; ○: Controls older than 80 years; △: AD patients younger than 80 years; □: AD patients older than 80 years.

2 subjects who died in the night period and who had the lowest amounts of AVP mRNA.

AVP mRNA in Relation to the State of AD

No significant correlation was found between age at onset of dementia and amount of AVP mRNA or total number of AVP mRNA expressing profiles (r = -0.42, p = 0.136; r = 0.32, p = 0.25, respectively). In addition, no correlation was observed between duration of AD and amount of AVP mRNA or total number of AVP mRNA expressing profiles (r = -0.23, p = 0.43; r = 0.32, p = 0.27, respectively). We could not find any relationship between the amount of AVP mRNA and cognitive impairment (FAST) (r = 0.21, p = 0.47), or with neuropathological changes in the cerebral cortex (r = 0.35, p = 0.22).

DISCUSSION

The suprachiasmatic nucleus (SCN) is considered to be the circadian pacemaker of the mammalian brain that coordinates hormonal and behavioral circadian rhythms (32). The SCN is affected in Alzheimer disease (AD), according to the typical cytoskeletal alterations that have been found in this structure (12, 33–34). Our previous studies showed that the numbers of AVP and vasoactive intestinal polypeptide immunoreactive neurons in the SCN decrease during aging and even more so in AD (11, 35). In the present study, we investigated the expression of AVP mRNA level in the human SCN. The in situ hybridization procedure on formalin-fixed paraffin-embedded material was improved to such a degree that we could, for the first time, visualize AVP mRNA expressing neurons in the human SCN and carry out quantitative measurements. A clear decrease of AVP mRNA was indeed found in the SCN of the AD patients. The total amount of AVP mRNA in the SCN, expressed as masked silver grains, was 3 times lower in AD patients than in age- and time-of-death-matched controls. Moreover, we found that the total number of profiles that expressed AVP mRNA in SCN in AD patients was only 40% of that of controls. It should be noted here that a loss of neurons expressing AVP-mRNA does not necessarily mean that the neurons have died; they could still be present but inactive and no longer express AVP mRNA. Activation of the SCN by bright light therapy in AD patients improves circadian rhythmicity (3). Our experiments in aged rats (9, 10) argue in favor of the idea that these
The decreased AVP mRNA levels in the SCN in AD patients corresponds well with previous reports showing a major reduction in the number of AVP immunoreactive neurons in the SCN (11–12). Since the SCN is the clock of the brain, the time of death should be considered as a possible confounding factor. We excluded this possibility by matching AD patients with control subjects who had died around the same time (Table 1). Moreover, the reduction in AVP mRNA level found in AD patients was present during the day, whereas no clear diurnal rhythm of AVP mRNA expressing neurons in the SCN was observed in AD patients. It is interesting to note that the day-night fluctuations in the amount of AVP mRNA and in the total number of profiles in the SCN were only observed in controls under 80 years of age. No diurnal fluctuations in AVP mRNA were observed in controls over 80 years. This finding also agrees with the disappearance of circadian rhythmicity in some AVP immunoreactive neurons in the SCN of elderly people (14).

The low amount of AVP mRNA is also reflected by a decreased number of AVP neurons in the SCN in subjects older than 80 years (11). Recently, a significant decrease of cerebrospinal fluid melatonin was found in the control subjects who were older than 80 years (36). We propose that degenerative changes in the SCN and a decrease in melatonin synthesis may underlie the common sleep disturbances among elderly people. In contrast to what was seen in the controls, no significant diurnal variations in any of the SCN parameters were observed in the AD group. Indeed, several studies showed the presence of circadian rhythm disturbances in aging and AD (3, 4, 14, 36).

The present study shows that these behavioral disturbances most probably have their basis in a decreased activity of the SCN in this disorder. AVP is one of the major neuropeptides in the SCN and is involved in the synchronization of the circadian rhythm of a light/dark cycle to the light entrainable oscillator (37). In addition, AVP may amplify the rhythm in this nucleus (38). On the basis of the observations in human and rat, one may expect that stimulation of the circadian system may have important therapeutic consequences for AD patients and elderly people. Indeed, exposure to bright light was found to have a positive effect on both the phase and amplitude of the circadian pacemaker (39–40). Appropriately timed exposure to bright light may thus be used in the treatment of circadian rhythm-related behavioral disturbances such as sleep disorders in AD patients and elderly people (41). There was no relationship between the amount of AVP mRNA in the SCN and age at onset of dementia, duration of AD and the severity of cognitive impairment (Table 2). However, it has to be mentioned that even when using the FAST, as in the present study, it may be difficult to clinically distinguish levels of dementia severity in the final stages of AD (21). In addition to Braak’s scores (25), a neuropathological estimate of the severity of AD was used (40–41) (Table 2). However, neither method revealed a relationship between the amount of AVP mRNA in the SCN and severity of AD pathology.

Depression is a common symptom in AD patients (27–28). Depression and dementia have a number of symptoms in common (42). In order to control for symptom overlap between dementia and depression we used the Cornell scale, which was specifically developed for the assessment of depression in all stages of dementia (23). In both AD and depression a relationship between the pathology of the SCN and dysfunction of biological rhythms may be present (43). Recently, we found a significantly lower amount of AVP mRNA in the SCN in depression (unpublished observation). Based on these findings, we considered the possibility that depressed AD patients might have the lowest amount of AVP mRNA, which turned out not to be the case. However, the amount of AVP mRNA or the total number of profiles with AVP mRNA in the SCN of AD patients with depression was not different from AD without depression. The reason could be that the AVP mRNA values in AD patients without depression were already extremely low. In conclusion, we found that, independent of the presence or absence of depression, AD patients showed a strongly decreased production of AVP in the SCN, which may be the biological basis of diurnal behavioral disorders and the beneficial effect of light therapy.

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