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

Liu, R-Y.

Citation for published version (APA):
Sleep impairment is one of the major side effects of glucocorticoid therapy. The mechanism responsible for this circadian disorder is not understood, but alterations in the suprachiasmatic nucleus (SCN) are presumed to play a major role. In the present study, the amount of vasopressin mRNA (AVP mRNA) expression in the SCN, one of its major neuropeptides, was investigated in 22 human subjects. The total amount of AVP mRNA expressed as masked silver grains in the SCN was two times lower in glucocorticoid-exposed patients (n=10; 5,115±1,314μm²) than in age- and clock-time-of-death-matched controls (n=10; 11,021±1,408μm²) (P=0.006). There was also a 53% decrease in the total number of profiles in the SCN that expressed AVP mRNA in glucocorticoid-exposed patients (16,759±3,110) compared with those in controls (31,490±3,816) (P=0.01). No significant correlation was found between the amount of AVP mRNA expression and the postmortem delay or brain weight in the controls or in the glucocorticoid-exposed group. In conclusion, glucocorticoids have an inhibitory effect on AVP mRNA expression in the human SCN, which may be the biological basis of the circadian rhythm disturbances during glucocorticoid therapy. This effect is rapid and reversible. Since vasopressin effects from the SCN inhibits the HPA-axis and cortisol inhibits vasopressin production in the SCN, the biological clock seems to be included in the physiology of the feedback system of the HPA-axis.
CHAPTER 6

Introduction
Glucocorticoid therapy potentially has numerous central side effects, such as insomnia and depression as well as impairment of memory and cognition, psychosis and convulsions (1-4). When subjects receive glucocorticoid therapy, insomnia occurs early and is usually unavoidable and sleep efficiency is reduced (1,2). The mechanism responsible for sleep disturbances in glucocorticoid-exposed patients is not known, but alterations in the suprachiasmatic nucleus (SCN) are presumed to be a key factor. The SCN is the major circadian pacemaker of the mammalian brain and coordinates hormonal and behavioral circadian rhythms (5). One of the endocrine rhythms regulated by the SCN is that of adrenal activity. The vasopressinergic neurons of the SCN are crucial in the regulation of this system. In the first place, the SCN-derived vasopressinergic projection to the dorsomedial and paraventricular nucleus of the hypothalamus (6) is responsible for the diurnal trough of adrenal activity. SCN lesions and microperfusion of vasopressin in rats have shown that this vasopressinergic projection inhibits the hypothalamic-pituitary axis (7). Degeneration of vasopressin (AVP) neurons of the SCN during the course of aging and in Alzheimer’s disease (8,9) is, therefore, thought to be an important causal factor for the elevated cortisol levels and for the decreased amplitude of circadian rhythms in this disorder (10,11). In the second place, several animal experimental studies have demonstrated that adrenalectomy and dexamethasone affect the levels of AVP or AVP mRNA in the SCN (12,13). The latter observations in rat suggest that the disorder of circadian rhythms in patients treated with glucocorticoids may be due to their action on the SCN. The present study was therefore performed on postmortem SCN of patients who had been exposed to glucocorticoids during the last premortem period. It shows for the first time that AVP mRNA expression in the human SCN is indeed suppressed by glucocorticoids.

Materials and Methods

Subjects
Ten subjects, who were free of glucocorticoid therapy during the premortem period and died from different diseases, served as controls. Of the ten patients who formed the glucocorticoid-exposed group, one had been exposed to high levels of endogenous glucocorticoids as the result of an adrenal tumor, and nine had been exposed to exogenous glucocorticoid tablet administration at different doses and duration during the premortem period until their death. For clinical pathological details see Table 1. Two subjects, who had received glucocorticoid treatment until 2 months before their death and one subject who had taken a low dose of beclomethason inhalation until his death, were evaluated separately. The patients in the glucocorticoid-exposed group had no pi-
<table>
<thead>
<tr>
<th>NBB</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>CTD (hours)</th>
<th>FD (days)</th>
<th>BW (g)</th>
<th>PMD (hours)</th>
<th>Diagnosis, Cause of death, Summary of the steroid medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>m</td>
<td>70</td>
<td>08.00</td>
<td>28</td>
<td>1,454</td>
<td>09.00</td>
<td>Lung cancer, Pneumonia, kidney insufficiency</td>
</tr>
<tr>
<td>P1</td>
<td>m</td>
<td>62</td>
<td>10.15</td>
<td>35</td>
<td>1,352</td>
<td>06.35</td>
<td>Adenocarcinoma with metastases, prednisone 30mg/day for last 18 days</td>
</tr>
<tr>
<td>C2</td>
<td>m</td>
<td>63</td>
<td>05.00</td>
<td>32</td>
<td>1,250</td>
<td>10.20</td>
<td>Myocardial infarction, lung emphysema, bronchopneumonia.</td>
</tr>
<tr>
<td>P2</td>
<td>f</td>
<td>65</td>
<td>02.40</td>
<td>91</td>
<td>1,132</td>
<td>14.20</td>
<td>Hypovolemic shock, dexamethasone 12 mg/day for last 5 months</td>
</tr>
<tr>
<td>C3</td>
<td>m</td>
<td>74</td>
<td>13.00</td>
<td>60</td>
<td>1,317</td>
<td>08.00</td>
<td>Myocardial infarction, diabetes</td>
</tr>
<tr>
<td>P3</td>
<td>m</td>
<td>71</td>
<td>10.00</td>
<td>41</td>
<td>1,385</td>
<td>06.00</td>
<td>Non small-cell lung carcinoma, hydrocortisone 200-300 mg/day for the last 3 months.</td>
</tr>
<tr>
<td>C4</td>
<td>m</td>
<td>39</td>
<td>00.30</td>
<td>130</td>
<td>1,400</td>
<td>16.30</td>
<td>Myocardial infarction.</td>
</tr>
<tr>
<td>P4</td>
<td>m</td>
<td>47</td>
<td>23.45</td>
<td>28</td>
<td>1,307</td>
<td>33.45</td>
<td>Lung carcinoma, prednisone 20mg/day for 13 days, stopped 2 days before death</td>
</tr>
<tr>
<td>C5</td>
<td>m</td>
<td>33</td>
<td>18.35</td>
<td>72</td>
<td>1,588</td>
<td>46.25</td>
<td>Car accident.</td>
</tr>
<tr>
<td>P5</td>
<td>m</td>
<td>36</td>
<td>05.08</td>
<td>42</td>
<td>1,750</td>
<td>28.37</td>
<td>Malignancy of the right testicle, dexamethasone 20 mg/day for 5 days</td>
</tr>
<tr>
<td>C6</td>
<td>f</td>
<td>60</td>
<td>08.54</td>
<td>87</td>
<td>1,102</td>
<td>08.06</td>
<td>Ovary carcinoma.</td>
</tr>
<tr>
<td>P6</td>
<td>f</td>
<td>69</td>
<td>14.30</td>
<td>120</td>
<td>1,074</td>
<td>08.30</td>
<td>Myocardial infarction.</td>
</tr>
<tr>
<td>C7</td>
<td>f</td>
<td>69</td>
<td>16.00</td>
<td>31</td>
<td>1,355</td>
<td>50.00</td>
<td>Sepsis, pneumonia. prednisone 40mg/day for the last 45 days</td>
</tr>
<tr>
<td>C8</td>
<td>f</td>
<td>58</td>
<td>07.45</td>
<td>28</td>
<td>1,221</td>
<td>06.45</td>
<td>Intracerebral haemotoma, postoperative coma.</td>
</tr>
<tr>
<td>P8</td>
<td>f</td>
<td>46</td>
<td>06.10</td>
<td>34</td>
<td>1,360</td>
<td>10.50</td>
<td>Adrenocortical carcinoma causing high levels of adrenal steroids [urinary 17-ketosteroids, 4164mmol/24h (normal, 21-52); 17-Hydroxy cortisol, 381 mmol/24h (normal, 10-52); plasma cortisone, 0.69mmol/L at 1000h and 0.77 mmol/L at 15.00h (normal, 0.14-0.55)]; preoperative corticosteroid supplement</td>
</tr>
<tr>
<td>C9</td>
<td>f</td>
<td>36</td>
<td>17.30</td>
<td>61</td>
<td>1,348</td>
<td>71.30</td>
<td>Faecal peritonitis, tetraplegia</td>
</tr>
<tr>
<td>P9</td>
<td>f</td>
<td>33</td>
<td>10.15</td>
<td>65</td>
<td>1,250</td>
<td>22.45</td>
<td>Progressive bronchial asthma, methylprednisolone minimal 30mg/kg for the last 2 days</td>
</tr>
<tr>
<td>C10</td>
<td>f</td>
<td>36</td>
<td>19.20</td>
<td>51</td>
<td>1,420</td>
<td>85.40</td>
<td>Multiple fractures, rupture of thoracic aorta</td>
</tr>
<tr>
<td>P10</td>
<td>f</td>
<td>31</td>
<td>22.30</td>
<td>31</td>
<td>1,499</td>
<td>04.45</td>
<td>AIDS stage IV, prednisolone 15mg/day; dexamethasone, 15mg/day for 30 days</td>
</tr>
<tr>
<td>P11</td>
<td>m</td>
<td>65</td>
<td>01.15</td>
<td>28</td>
<td>1,067</td>
<td>09.10</td>
<td>Carcinoma of the right vocal cord, chronic low dose beclomethasone inhalation and 200 μg/day for last 7 days</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>72</td>
<td>13.50</td>
<td>34</td>
<td>1,216</td>
<td>06.25</td>
<td>Cardiac failure with respiratory insufficiency, Cachexia. Chronic Prednisone use, doses of 5-30mg/day for the last 4 months, stopped 2 months before death</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>63</td>
<td>17.01</td>
<td>27</td>
<td>1,216</td>
<td>06.25</td>
<td>Mammary carcinoma, dexamethasone 5mg/day for ten days, stopped 2 months before death</td>
</tr>
</tbody>
</table>

tuitar disorder as an indication for corticosteroid therapy. After their death, brain autopsy was performed on the patients and controls as part of the program of the Netherlands Brain Bank. Written informed consent for brain autopsy and the use of the tissue and medical records for research purposes were obtained before subjects entered the study. The brains of the corticosteroid-treated patients were matched with the 10 controls for age, sex and clock time of death (Table 1).

Measurement of AVP mRNA in SCN
In situ hybridization was performed on every fiftieth (6-μm) section of the SCN as described extensively before (9). Briefly, the AVP probe (hvp3) consisted of an oligomer of 48 nucleotides complementary to bases 411-458 of the preprovasopressin precursor (14). The specificity of the probes has been described previously (9,15). The probe was 3'-end labeled using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α-35S]dATP as described earlier (16). Each section was incubated with 68 μl hybridization solution containing an approximate 1 x 10^6 cpm labeled probe. After overnight incubation at 42 °C, the sections were rinsed in 1 x SSC for 30 min at 50°C, 2 x 30 min 0.1 x SSC at 50°C, and 2 x 30 min 0.1 x 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 Detol 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 SONY XL-77CE videocamera mounted on a Zeiss microscope. The microscope was equipped with plan-neofluar objects and a scanning stage. The main principle and procedure of the IBAS measurement have been extensively described before (9,16). Briefly, the area of the SCN was manually outlined at low magnification (2.5x objective) and a grid of fields corresponding to a 40 x objective was superimposed. From this grid 50% of the fields indicated in red rectangles were randomly selected and the 40x objective images were stored. Then, at high magnification (40x objective), each field was retrieved at high resolution on the image analysis monitor. A mask was superimposed on the silver grains in their images. The total mask covering the silver grains in these profiles was calculated under program control. The silver grains of the background were subtracted. Every fiftieth section through the entire SCN was measured and stored in the IBAS. Finally, the total number of profiles express-
CHAPTE RR  6

ing AVP mRNA in the SCN and the total mask of the silver grains over the profiles were
calculated as an estimate of total amount of AVP mRNA expression in the SCN.

Statistical Analysis
Differences in amount of AVP mRNA in the SCN or total number of AVP mRNA ex-
pressing cells between the groups were tested using the Wilcoxon paired matched pairs
test. Correlation of brain weight, postmortem interval and fixation time versus amount
of AVP mRNA was analyzed by Spearman correlation coefficients in the glucocorticoid-
exposed group and controls. A significance level of 5% (two-tailed) was used in all sta-
tistical tests. Throughout this paper, values are expressed as mean ± standard error of
the mean (SEM).

Results

Decreased total amount of AVP mRNA in glucocorticoid exposed patients
The in situ hybridization procedure on formalin-fixed paraffin-embedded material al-
lowed us to show and quantify AVP mRNA expressing cell profiles in the human SCN of
all patients. A clearly decreased amount of AVP mRNA expression was found in the SCN
of the majority of the patients in the glucocorticoid-exposed group (Figure 1A, 1B). The
total mask of silver grains in cells (an estimate of total amount of AVP mRNA in the
SCN) was reduced by 46% in glucocorticoid-exposed patients (5,115±1,314μm²) as com-
pared with controls (11,021±1,408μm²) (P=0.006) (Fig 2). There was also a 53% de-
crease in the total number of profiles in the SCN that expressed AVP mRNA in glucocor-
ticoid-exposed patients (16,759±3,110) compared with those in controls (31,490±3,816)
(P=0.01). Since the SCN is the clock of the brain, the time of death should also be con-
sidered a possible confounding factor. We excluded this possibility by matching gluco-
corticoid exposure patients as much as possible with control subjects who had died
around the same time (Table 1). Moreover, the differences between controls and gluco-
corticoid- exposed patients were found at every time point over the entire period of the
day and night (Fig. 3).

The doses and duration of glucocorticoids in relation to the AVP mRNA expression in the
SCN: case studies
During the highest cumulative doses of glucocorticoid administration in our study, which
was 12 mg of dexamethasone per day for the last 5 months (patient #99096), the total
amount of AVP mRNA expression and the total number of profiles were around the
mean value of the cortisol-exposed group (6,522μm² and 11,771, respectively).
The total amount of AVP mRNA expression in the SCN for two subjects (patient # 95092

101
Fig. 1 Thionin-counterstained emulsion autoradiograms in frontal sections (6µm) 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: Corticosteroid exposed patient. Note there were fewer AVP mRNA expressing neurons in corticosteroid exposed patients. Scale bar=20µm.
Fig. 2 Estimated total amount of AVP mRNA in the SCN (expressed as masked area of silver grains) of the controls and the corticosteroid-exposed subjects (CST). The bars and error lines represent the mean and standard error of the mean (SEM).

Fig. 3 Day-night fluctuation in the total amount of AVP mRNA of the SCN in controls and in the glucocorticoid-exposed subjects (CST). Note that at any moment of the day the values for CST are lower than those of controls.
and 95054), who stopped glucocorticoid treatment 2 months before death was 13,394µm² and 8,694µm², respectively, whereas the total number of profiles that expressed AVP mRNA in the SCN was 19,893 and 21,606, respectively, all data in the range of the controls.

The suppressing effect of prednisone on AVP mRNA expression in the SCN became already obvious on the second day of administration, since the total amount of AVP mRNA and the total number of profiles in the SCN of patient # 84026 were only 97µm² and 709 respectively. The patient who received a low dose of beclomethason inhalation for the last 7 days (patient #95051) did, however, not show any difference as compared with controls, either in the total amount of AVP mRNA expression (12,182µm²) or in the total number of cell profiles (14,703).

**AVP mRNA expression in the SCN in relation to age, postmortem delay and fixation time**

There were no statistical differences between the glucocorticoid-exposed group and the control group in postmortem delay (P=0.28), brain weight (P=0.95), fixation time (P=0.79) or age (P=0.96). No significant correlation was found between the amount of AVP mRNA and postmortem delay in controls or the glucocorticoid-exposed group (r=0.39, P=0.25; r=-0.13, P=0.70; respectively). Neither in controls, nor in corticosteroid treated patients was a correlation found with brain weight (r=0.20 P=0.61; r=0.19, P=0.60, respectively). In a pilot study we found an effect of long fixation times on the amount of AVP mRNA expression in the SCN. To further substantiate this observation, two corticosteroid-exposed patients with long fixation times (patients # 93095; 618 days and patient #93016; 269 days) were compared with controls who had long fixation times (control #96426; 728 days and control #96268 798 days). The results showed that long fixation times indeed sharply reduced the signal of AVP mRNA expression in the SCN. Corticosteroid-exposed patients with long fixation times (more than 130 days) were therefore excluded from the present study.

**Discussion**

During glucocorticoid (GC) therapy patients frequently suffer from sleep-wake rhythm disturbances (1,4), but the underlying mechanisms of this side effect are not known. In the present study, a strongly decreased amount of AVP mRNA expression was found in the SCN of the glucocorticoid-exposed group. The total number of cell profiles that expressed AVP mRNA in the SCN was also strongly decreased in glucocorticoid-exposed patients compared with those in controls, indicating that not only the total AVP production but also the AVP production per cell was diminished. The SCN is considered to be the circadian pacemaker of the mammalian brain and to coordinate hormonal and behavioral circadian rhythms (5). AVP is one of the major neuropeptides in the SCN.
and is involved in the amplification of the amplitude of circadian rhythms (17) and in the synchronization of the circadian rhythm of a light/dark cycle to the light entrainable oscillator (18). Isobe et al. reported that in rat the AVP content in the SCN and plasma corticosterone level were inversely correlated, although the causality of this relationship did not become clear from that study (12). The increased release of AVP from SCN terminals during the light period in rat and monkey coincides with the low levels of circulating corticosterone at this time of the day, and the AVP neurons of the SCN are considered to be a major inhibiting factor for the CRH neurons in the PVN (19,20). An opposite relationship between a high activity of CRH neurons of the PVN and a decreased activity of the AVP neurons in the SCN was observed in aging humans and Alzheimer patients where the increased CRH mRNA level in the PVN (21) and cortisol in the CSF (10) go together with a pronounced decline of AVP activity in the SCN (8,9,22). Sleep disruption, nightly restlessness and other circadian rhythm disturbances are indeed frequently seen in aging people and even more so in Alzheimer patients (11,23-25). Clinical research indicates detrimental effects of corticosteroids on sleep architecture. Turner and Elson report insomnia and nightmares in cancer patients receiving 8-16mg dexamethasone daily (26). The study by Young et al (27) demonstrated statistically significant reductions in the percentage of REM sleep and number of REM periods following 20mg of hydrocortisone orally, twice daily.

The neurobiological basis of the disturbed sleep architecture in aging, Alzheimer’s disease and during corticosteroid exposure was hypothesized to be a decreased functional activity of the SCN. Only a few animal studies have so far demonstrated that adrenal steroids may affect the AVP content of the SCN. Adrenalectomy as well as treatment of the rats with dexamethasone has shown that the expression of AVP mRNA in the SCN is susceptible to alterations in circulating levels of glucocorticoids, at least during a narrow time window in the diurnal cycle (13). Boyer et al (1979) found that there is a persistently abnormal cortisol circadian rhythm in patients with Cushing’s disease (28). These observations and our present data support the hypothesis that glucocorticoid exposure during corticosteroid treatment or in Alzheimer’s disease could cause circadian rhythm disturbances because they affect the function of the SCN. Whether or not this is a receptor-mediated effect, and whether the SCN is directly or indirectly influenced by corticosteroids, should be further investigated.

We did not find an age-related decline in AVP mRNA in the SCN in the present study. This is probably due to the relatively young average age of the subjects in our study, which was 53±5 years. This is much younger than the 80 years of age in which previous studies found a change in the number of neurons expressing AVP peptide and the amount of AVP mRNA in the SCN (8,9). Corticosteroid seems to have a suppressive effect on various peptidergic neurons in the hypothalamus. A recent report showed a negative correlation between circulating cortisol levels and metabolic activity of the hypothalamus.
Corticosteroids seem to have a global inhibiting effect on brain metabolism as well (29). Earlier studies by our group have shown that corticosteroid decreased the amount of CRH and AVP in the paraventricular nucleus and AVP in the supraoptic nucleus of the human hypothalamus. On the other hand, oxytocin immunocytochemistry in the PVN was not affected by corticosteroids, indicating specificity in the effect of corticosteroids on hypothalamic neurons (31).

The suppressive effect of prednisone on AVP mRNA expression in the SCN seems to act rapidly since its effect was already obvious on the second day of administration (patient # 84026). On the other hand, the patient who had received low dose beclomethason inhalation for the last 7 days (patient #95051) did not show any difference as compared with controls, either in the amount of AVP mRNA expression or in the total number of cell profiles containing AVP mRNA in the SCN, while the same patient showed a decrease in CRH and AVP content in the PVN and SON (31). The SON and PVN thus seem to be more sensitive to the effect of beclomethason than the SCN. In a study of low dose (0.5mg) oral dexamethasone in depression, there was no change in sleep structure (32). Whether these results can be explained by the fact that AVP expressing cells in the PVN are more sensitive to glucocorticoids than AVP-expressing cells in the SCN should be further investigated. On the other hand, during the highest cumulative doses of glucocorticoid administration in our study, which was 12mg/day dexamethasone for the last 5 months (patient # 99096), AVP mRNA expression was still detectable around the mean value of the cortisol-exposed group, suggesting some mechanism of partial adaptation to a long-term, high dosage of corticosteroids. The amount of AVP mRNA was in the range of control values 2 months after stopping prednisone therapy, suggesting recovery from suppression (patient # 95054 and 95092). This agrees with an animal experimental study that showed that one dose of dexamethasone did not have an effect on the AVP content of the SCN on the next day (12). The inhibiting effect of glucocorticoids on the AVP mRNA in the SCN is of special interest in depression. In this disorder high cortisol levels and sleep disorders are often found. These may well be explained by the inhibitory effect of glucocorticoids on the SCN. Indeed, we recently found a decreased activity of AVP neurons in the SCN in depression (Zhou et al., 2000 submitted). In conclusion, the glucocorticoids have an inhibitory effect on the AVP mRNA in the SCN. This effect is rapid and reversible. Since vasopressin efferents from the SCN inhibit the HPA-axis (7,33,34) and corticosteroids inhibit the vasopressin production of SCN neurons, the vasopressin neurons of the SCN seem to be incorporated in the feedback system of the HPA-axis.

Acknowledgments

We are grateful to J.J. van Heerikhuize and B. Fisser for their technical assistance, to W.T.P. Verweij for secretarial help, to Michel Hofman for his kind help with statistical evalua-
tion and to C. Pool for his IBAS image analysis programs. Brain material was obtained from the Netherlands Brain Bank (coordinator R. Ravid). This study is partly supported by the Royal Netherlands Academy of Arts and Sciences (98CDP004; R-Y Liu and J-N Zhou), by the Van den Houten foundation (Zhou), by PAD 9607 (D.F. Swaab), and by the National Key Project for Basic Research of China (G1999054007).

References


