

Alterations in Arginine Vasopressin Neurons in the Suprachiasmatic Nucleus in Depression

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Background: Circadian rhythm disturbances are frequently found in depressed subjects. Although it has been presumed that these disturbances may reflect a disorder of the circadian pacemaker, this has never been established. The suprachiasmatic nucleus (SCN) is the pacemaker of the circadian timing system in mammals, and arginine vasopressin (AVP) is one of its major neuropeptides. As peptide content is often taken as a measure for activity, we hypothesized that a decreased number of AVP-immunoreactive (AVP-IR) neurons and amount of AVP-messenger RNA (mRNA) would be present in the SCN of depressed subjects.

Methods: Brains of 11 subjects suffering from major depression (8 cases) and bipolar disorder (3 cases), and of 11 controls, matched for sex, age, and clock time at death, were collected. The number of AVP-IR neurons in the SCN was determined by means of a digitizer (CalComp Inc, Reading, England). The amount of AVP-mRNA expression in the SCN was quantified with the

Interaktive Bild Analyse System image analysis system (Kontron, Munich, Germany).

Results: In depressed subjects, the number of AVP-IR neurons in the SCN was more than one and a half times higher than in controls, while the total masked area of silver grains, as an estimate of the amount of AVP-mRNA, was about one half that of controls.

Conclusions: Contrary to our hypothesis, an increase in the number of AVP-IR neurons in the SCN in depression was found, together with an expected decrease in AVP-mRNA. These findings suggest that, in depressed patients, both the synthesis and release of AVP in the SCN is reduced, resulting in an impaired functional ability. A disbalance between AVP production and transport needs further investigation in future studies.

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THE SUPRACHIASMATIC nucleus (SCN) is the circadian pacemaker of the mammalian brain, generating and coordinating diurnal rhythms (eg, sleep-wakefulness, body temperature, hormonal rhythms).^{1,2} Over the years, a variety of studies have pointed to the possible involvement of the circadian pacemaker in depression.³⁻⁵ An argument in favor of this idea is that in the melancholic type of depression, patients feel worst in the morning and typically suffer from early morning awakenings.³⁻⁵ In addition, a decrease in the amplitude of body temperature is consistently found in depressed patients.^{4,5} Furthermore, the successful treatment of seasonal affective disorder with light therapy,^{6,7} and, to a lesser extent, also of patients with non-seasonal affective disorders,⁸ has led to the hypothesis that the effect of bright light on depression acts on the circadian pacemaker via the retinohypo-

thalamic tract.^{3,4,6,9} Whether the observed disturbances of circadian rhythms in depression indeed reflect a disorder of the SCN has, however, so far not been established.

Another important hypothalamic structure that is involved in depression consists of the corticotropin-releasing hormone (CRH) neurons of the paraventricular nucleus (PVN). The increased number of neurons expressing CRH and the increased amount of CRH-messenger RNA (mRNA) are signs of strong activation of these neurons in depression.^{10,11} These findings are of particular interest because there are similarities between the signs and symptoms of major depression (MD) and the behavioral effects of centrally administered CRH in animals¹² and CRH overproduction in transgenic mice.¹³ Furthermore, there is a functional relationship between arginine vasopressin (AVP) in the SCN and CRH in the PVN. Arginine vasopressin neurons from the SCN inhibit

SUBJECTS AND METHODS

SUBJECTS

Brains of 11 depressed subjects were collected and matched with 11 controls for sex, age, and clock time at death (**Table**). Brain material of both depressed and control subjects was obtained from the Netherlands Brain Bank. Within the framework of the Netherlands Brain Bank autopsies take place after informed consent is given by the donor and/or the next-of-kin for the following: (1) performing a brain autopsy, (2) the subsequent use of the tissue and fluids obtained for scientific research, and (3) permission to use the donor's medical history for research purposes. The medical records of the control subjects did not report any psychiatric or neurological disease, except for subject C2b. The diagnosis was established by the physician in attendance and confirmed by a psychiatrist (W.J.G.H), after reviewing the medical record. The DSM-IV¹⁵ criteria were used for the diagnosis of MD and bipolar disorder (BD), at any time during life. No relatives were interviewed to give additional information to the medical record diagnosis. In case data were missing, an additional interview took place with the physician who treated the subject. In this procedure, DSM-IV criteria for the presence, duration, and severity of symptoms of either MD or BD, as well as the exclusion of other psychiatric and neurological disorders, were systematically scored. Eight patients fulfilled the criteria for MD and 3 fulfilled the criteria for BD (Table). Four MD subjects and 2 BD subjects suffered from their last episode just before death. In the 2 BD cases, this last episode was a manic episode. For detailed information on the time of the last episode, see the Table. A complete overview of the psychiatric medication in the past and in the last month before death for both depressed and control subjects is given in the Table. The medical record did not reveal any alcohol or other drug abuse among depressed subjects or control subjects at the time of death, but no toxicology screens were performed. Microscopical examination of the liver of subject D11 showed microabscesses and infiltration with neutrophilic and eosinophilic granulocytes. These signs could

be compatible with drug intoxication. Potential cases and controls were excluded if not enough material was available to stain the complete SCN. For this reason, 3 controls of the immunocytochemical study were replaced by 3 other controls for the in situ hybridization (ISH) study (C2, C7, and C9) (Table).

IMMUNOCYTOCHEMISTRY AND MORPHOMETRY

For immunocytochemical analysis of AVP, 6- μ m-thick paraffin sections through the entire SCN were stained with an antibody against AVP. The immunocytochemical and morphometric procedures were performed, as described extensively elsewhere.¹⁶⁻²¹ Briefly, measurements of the vasopressinergic SCN area and the number of cell nuclei were performed unilaterally by means of a digitizer (CalComp Inc, Reading, England). The rostrocaudal axis was determined by staining every 25th section, starting from the lamina terminalis and ending at the caudal end of the optic chiasm. The rostral and caudal borders of the SCN were assessed by staining every 10th section in the area and by determining the sections in which, respectively, the first and the last AVP cells were present. The volume of the SCN was determined by integrating all the area measurements of the SCN sections that contained immunocytochemically stained cells. The numerical cell density of AVP-IR neurons was estimated by counting the total number of nuclear profiles per unit area, followed by a discrete "unfolding" procedure, which included the modification proposed by Cruz-Orive²² and a correction for section thickness (6 μ m, z-axis). All nuclear profiles within a rectangular grid in one of the oculars that corresponded to 38000 μ m² in the section were measured according to Gundersen.²³ The total number of AVP-IR neurons was computed by multiplying the numerical cell density with the corresponding volume of the AVP subnucleus.

ISH AND QUANTITATIVE ANALYSIS

For ISH, 3 control subjects (C2, C7, and C9) were replaced by other matched controls (Table) because not

CRH neurons in the PVN of rats.¹⁴ In this way, the SCN plays a key role in the circadian regulation of the hypothalamic-pituitary-adrenal axis resulting in circadian fluctuations of cortisol levels.

On the basis of these observations, we hypothesized that the functional ability of the SCN in maintaining normal biological rhythms might be diminished in depression. Our hypothesis was that the number of AVP containing neurons and the amount of AVP-mRNA in the SCN of depressed subjects would be decreased.

RESULTS

The groups were matched for sex, age, and clock time at death. Both groups consisted of 4 female and 7 male subjects. Data on age (67 \pm 8.7 years, for depressed subjects; 70 \pm 12 years, for control subjects); brain weight (1280 \pm 162.3 g, for depressed subjects; 1399 \pm 307.5 g, for control subjects) (P = .96); clock time at death; PMD; and fixation time (38 \pm 12.5 hours, for depressed sub-

jects; 34.4 \pm 15.5 hours, for control subjects) (P = .48) are presented in the Table. There were no differences in these factors between the control and depression group except for the PMD. The control group had a shorter mean \pm SD average PMD (9.5 \pm 4.7 hours) than the depression group (32.5 \pm 20.4 hours) (Z = -2.67, P = .008), but no significant relationship was found between PMD and the number of AVP-IR neurons (r_s = -0.20, P = .56, for the depressed subjects; r_s = 0.34, P = .31, for the controls) or amount of AVP-mRNA (r = 0.15, P = .66, for the depressed subjects; r_s = 0.20, P = .55, for the controls).

The mean \pm SD of AVP-IR neurons in depression (6589 \pm 2389) was found to be significantly higher than in controls (3706 \pm 1678) (Z = -2.40, P = .02) (**Figure 2**). A clearly smaller amount of AVP-mRNA was found in the SCN of the subjects with depression (Figure 2). In depressed subjects, the total mask area of silver grains, as an estimate of total amount of AVP-mRNA in the SCN, was approximately half that of control subjects (5921 \pm 3802 μ m² vs 12206 \pm 5827 μ m²) (Z = -2.49;

enough material was left to stain the entire SCN. Hybridization was performed on every 50th section of the SCN. Sections were randomly divided over 2 hybridization assays of approximately 120 sections each. The AVP probe (human vasopressin 3, provided by G. Mengod and J. M. Palacios, Basel, Switzerland) consisted of an oligomer of 48 nucleotides complementary to bases 411 to 458 of the human pre-vasopressin precursor.²⁴ The specificity of the probe has been described previously.^{25,26} The probe was 3'-end labeled using terminal deoxynucleotidyl transferase (Roche, Mannheim, Germany) and [α -³⁵S] dATP (NEN Life Sciences, Boston, Mass) as described earlier.²⁷ Tissue pretreatments were performed mainly as previously described²⁷ except for the deproteinization and delipidation. Deproteinization was done in proteinase-K (10 μ g/mL at 37°C) for 15 minutes instead of 30 minutes. Delipidation was performed in 0.1% Triton X-100 (Sigma, St Louis, Mo) in phosphate-buffered saline for 10 minutes and sections were washed in phosphate-buffered saline without dehydration before hybridization. Each section was incubated with 68- μ L hybridization solution containing approximately 1×10^6 cpm-labeled probe. After overnight incubation at 42°C, the sections were rinsed in $1 \times$ silver sulfadiazine chlorhexidine for 30 minutes at 50°C, 2×30 minutes $0.1 \times$ silver sulfadiazine chlorhexidine at 50°C, and 2×30 minutes $0.1 \times$ silver sulfadiazine chlorhexidine at room temperature. Sections were dehydrated at room temperature in 300-mmol ammonium acetate (pH, 5.5)/100% ethanol at volume ratios 1:1, 3:7, 1:9, and 0:1. To check the autoradiographic signal, a β -max hyperfilm (Nycomed Amersham plc, Buckingham, England) was apposed and developed after 5 days. Subsequently, slides were dipped in photographic emulsion (NTB2; Eastman Kodak, Rochester, NY) at 42°C, dried on a cool glass plate, and stored in a light-tight box at 4°C. After 17 days, slides were developed for 2 minutes in Dektol Developer (Sigma, St Louis, Mo) at 15°C and fixed in Kodak fixer (Sigma) at 15°C for 10 minutes. Sections were washed to remove the fixative and counterstained with thionine.

For quantitative analysis of the ISH signal of the AVP-mRNA in the SCN, the Interaktive Bild Analyse System image analysis system (Kontron, Munich, Germany) was

connected to a Bosch TYK9B television camera (Bosch, Stuttgart, Germany) equipped with a chalnicon tube mounted on a Zeiss microscope (Zeiss, Munich). The microscope was equipped with planapo objectives, a blue filter, and a scanning stage. The main principle and procedure of the Interaktive Bild Analyses System measurement have been extensively described before.²⁸ Briefly, the area of the SCN was manually outlined at low magnification ($\times 4$ objective) and a grid of fields was superimposed. From this grid, 50% of the fields indicated in red rectangles were randomly selected and stored (**Figure 1A**). Then, at high magnification ($\times 40$ objective), each field was retrieved on the image analysis monitor (Figure 1B). A mask was superimposed over the silver grains in these images. After the blue filter was removed, the profiles identified as cells by means of thionin staining were manually outlined. Finally, the total number of profiles expressing AVP-mRNA in the SCN and total mask area of the silver grains in the profiles were calculated as an estimate of total amount of AVP-mRNA in the SCN. In addition, total mask of the silver grains was divided by the total number of profiles to estimate the mean amount of AVP-mRNA per profile. This gives an estimate of the average AVP-RNA production per neuron.

Neither for the assessment of the number of AVP-IR neurons nor for the quantification of the AVP-mRNA were the raters blind to the antemortem diagnosis, but the measurements were standardized in such a way that this could not have influenced the study outcome.

STATISTICAL ANALYSIS

Differences among the groups were statistically evaluated by the Wilcoxon signed-rank test (2-tailed). Values of $P < .05$ were considered to be significant. All values are expressed as mean \pm SD. Differences within the depressed group according to their medication in the last month were tested with the nonparametric Mann-Whitney *U* test.

Linear regression analysis was performed to study the effects of postmortem delay (PMD) and the duration of the disease on the AVP data set, using the Spearman correlation coefficient.

$P = .01$). Furthermore, the mean \pm SD area of masked silver grains per profile was significantly lower in depressed subjects ($0.33 \pm 0.11 \mu\text{m}^2$) compared with control subjects ($0.52 \pm 0.15 \mu\text{m}^2$) ($Z = -2.85$, $P = .004$). Although there was a tendency toward a lower mean \pm SD number of profiles that expressed AVP-mRNA in the SCN in the depressed subjects (16072 ± 8036) than in the controls (23372 ± 8202), this difference did not reach significance ($Z = -1.87$, $P = .06$).

There was no difference either in the number of AVP-IR neurons ($Z = -0.57$, $P = .65$) or in the amount of AVP-mRNA ($Z = -0.95$, $P = .41$) between 4 subjects who had taken lithium in the past (D1, D4, D7, and D11; D1 and D7 took lithium in the last month before death), and the other depressed subjects. In addition, we did not find any difference in the number of AVP-IR neurons ($Z = -0.38$, $P = .79$) or AVP-mRNA ($Z = -0.57$, $P = .65$) between the subjects who took benzodiazepines (D4, D5, D7, D8, D9, D10, and D11) during the last month before death and the other subjects. The number of AVP-IR neurons and AVP-

mRNA in 3 subjects who were treated with selective serotonin reuptake inhibitors during the last month before death (D2 with fluoxetine, D5 with fluvoxamine, and D10 with paroxetine) did not differ from the other subjects ($Z = -0.612$, $P = .63$, and $Z = -0.41$, $P = .78$, respectively).

There was no relationship between either the number of AVP-IR neurons and the duration of the disease (from <1 year to 51 years) ($r_s = 0.04$, $P = .89$), or for the amount of AVP-mRNA and the duration of the disease ($r_s = 0.009$, $P = .98$).

The differences in the number of AVP-IR neurons and the amount of AVP-mRNA between MD subjects and matched controls did not change in significance when the BD subjects were left out of the analysis ($Z = -2.52$, $P = .01$, and $Z = -2.10$, $P = .04$, respectively).

COMMENT

In the present study, we found that the number of AVP-IR neurons in the SCN was higher in depressed subjects than

Brain Material of Depressed and Control Subjects*†

Subject	Sex	Age/Age at Onset, y	Brain Weight, g	PMD, h	Fix, d	Time at Death	Month of Death	Cause of Death	No. of Episodes/End of Last Episode‡, No. of Months Before Death	Past Suicide Attempt§	Psychiatric Medication Taken	
											In the Last Month	In the Past
D1	M	51/41	1390	75	28	2 PM	November	Respiratory insufficiency, lung emphysema	8/7	No	LI, HAL, PHT ¶	BZD
C1	M	49	1254	22	33	3:10 PM	November	Sepsis, colon carcinoma			BZD ¶	
D2	F	55/≤40	1320	7	52	7:45 AM	November	Heart failure, urosepsis	2/Death	Yes	SSRI, BRO	MAP, BZD, TCA, PHT
C2a	F	50	1210	7	40	7 AM	January	Renal insufficiency, multiple myeloma				
C2b#	F	58	1221	7	28	7:15 AM	March	Postoperative coma after craniotomy				
D3	M	61/≤50	1424	41	35	4:40 AM	October	Pneumonia	1/144	No	PHT	TCA
C3	M	63	1250	10	32	5 AM	January	Pneumonia				
D4	M	63/12	1210	20	33	2:15 PM	March	Heart failure	>6/Death	No	HAL, BZD, PHT	LI
C4	M	78	1442	8	24	12:15 PM	July	Cardiac arrhythmia				
D5	M	70/40	1500	44	28	7 PM	December	Heart failure	4/Death	No	SSRI, BZD, CLZ	
C5	M	70	1454	9	33	8 AM	February	Pneumonia, renal failure				
D6	M	71/53	975	16	38	4:15 PM	February	Cerebral ischemia, pneumonia	4/Death	Yes	None	BZD, MAO-I, TCA
C6	M	74	1317	8	60	1 PM	November	Heart failure, myocardial infarction				
D7	M	71/≤65	1109	14	26	10:30 PM	August	Respiratory insufficiency	≥2/21	No	LI, BZD, MAOI, PHT	
C7a	M	85	1400	16	44	4:50 PM	July	Chronic myelocytic leukemia				
C7b#	M	61	2220	14	64	9:22 PM	April	Esophagus carcinoma				
D8	F	72/54	1287	22	39	7 PM	January	Pneumonia	≥3/36	No	BZD	MAP
C8	F	63	1216	6	32	5:01 PM	September	Mammary carcinoma, euthanasia			BZD¶	BZD
D9	F	72/53	1116	28	35	4:20 AM	April	Heart failure, septic shock, pyelonephritis	≥4/108	No	BZD	MIA, TCA
C9a	F	73	1344	8	34	9:10 AM	February	Septic shock, pneumonia				
C9b#	F	65	...	7	28	1:45 AM	February	Respiratory insufficiency				BZD
D10	M	74/74	1444	57	35	5:05 PM	March	Strangulation (suicide)	1/Death	Yes	ZUC, SSRI, BZD	None
C10	M	78	1440	7	32	4 AM	September	Heart failure, lung embolism				
D11	F	80/60	1300	33	69	8 AM	December	Pneumonia	≥4/Death	No	LI, HAL, ZUC	TCA, BRO, PHT, BZD, CAR
C11	F	78	1135	6	32	8 AM	November	Respiratory insufficiency, lung carcinoma			BZD	BZD

*PMD indicates postmortem; Fix, fixation time; D, depressed subject; M, male; LI, lithium; HAL, haloperidol; PHT, phenothiazine; BZD, benzodiazepine; C, control subject; F, female; SSRI, selective serotonin reuptake inhibitors; BRO, bromperidol; MAP, maprotiline; TCA, tricyclic antidepressants; CLZ, clozapine; MAOI, monoamine oxidase inhibitor; MIA, mianserin; ellipses, not determined; ZUC, zuclopenthixol; and CAR, carbamazepine.

†All patients suffered from major depression, except D1, D4, and D11, who had bipolar disorder.

‡All last episodes were depressive, except for subjects D4 and D11, whose last episode was a manic episode.

§Subject D2 attempted suicide 1 month before death, D6 7 days before death, and D10 died of the attempt.

¶Also used corticosteroids.

||Also used morphine.

#Because not enough material was available, these subjects replaced the "a" subjects for in situ hybridization.

in control subjects. At the same time, the expression of AVP-mRNA in the SCN was lower in depressed subjects compared with control subjects. The difference in AVP-mRNA is at least partly caused by a decrease in the mean

AVP production per neuron. These findings indicate a change in the balance between the production and transport of AVP in depression. A functional alteration of neurons in the SCN is in line with circadian rhythm distur-

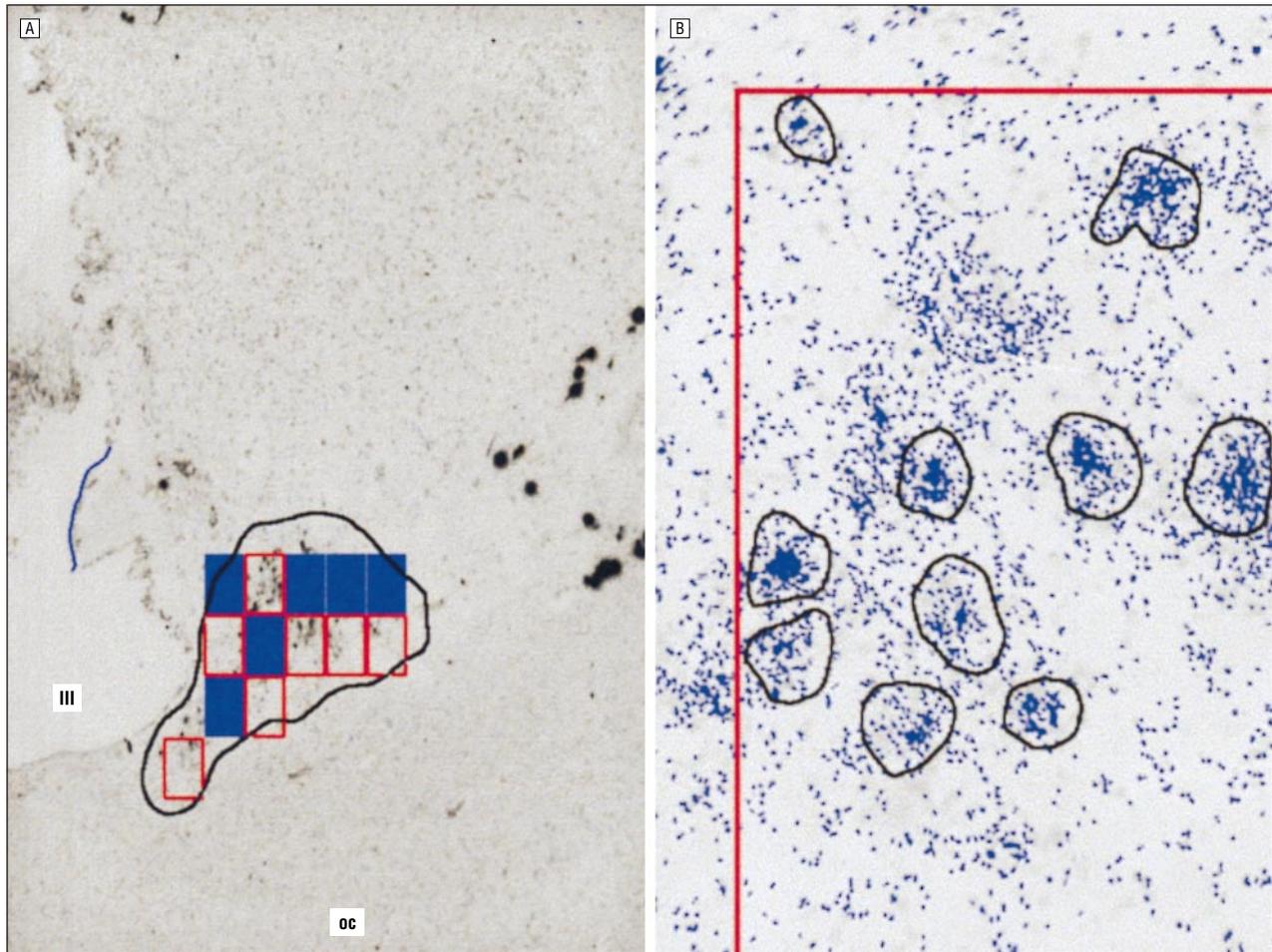


Figure 1. A, Outline of the suprachiasmatic nucleus at low magnification ($\times 2.5$ objective) at the Interaktive Bild Analyses System monitor with superimposed grid and selected fields. B, Outline of positive profiles at high magnification ($\times 40$ objective) at the Interaktive Bild Analyses System monitor with superimposed mask over the silver grains and red inclusion line.

bances that have been found in depression (ie, in sleep-wakefulness, body temperature, hormonal rhythms, and the periodicity of manic-depressive cycles in some BP patients).^{4,5}

As mentioned in the introduction, we hypothesized that the number of AVP-IR neurons in the SCN would be decreased. This would be in line with an attenuated inhibition of AVP from the SCN on CRH neurons in the PVN,¹⁴ which could explain the increased number of CRH neurons together with increased CRH-mRNA levels in the PVN.^{10,11} Finally, this would lead to the frequently found increased levels of cortisol in depression. It was thus a surprise to find just the opposite, namely an increased number of AVP-IR neurons in the SCN in depression. We then wanted to know whether this increase was also reflected by the production of AVP in these neurons and performed an ISH for AVP-mRNA. The results of this experiment brought us back to our hypothesis, because we found a clearly decreased amount of AVP-mRNA in depression. Probably there is accumulation of AVP in the neurons of the SCN in depression caused by a decreased transport rate of the neuropeptide. Arginine vasopressin is normally transported from the SCN to its target areas by axonal transport. So far, there is not much known about changes in transport rate

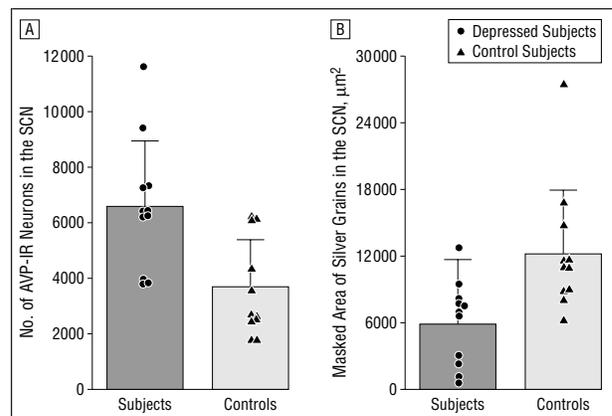


Figure 2. The number of arginine vasopressin-immunoreactive (AVP-IR) neurons (A) and the mask area of silver grains of the AVP-messenger RNA (B) in the suprachiasmatic nucleus (SCN) in control subjects ($n=11$) and depressed subjects ($n=11$). The error bars indicate the SD. Note the change in the balance between the presence of more AVP and less AVP-messenger RNA in depression.

related to psychiatric diseases, but in Alzheimer disease, a decreased axonal transport rate of the neurotrophin/track complex caused by cytoskeletal changes may be the underlying event for the neuronal atrophy in the

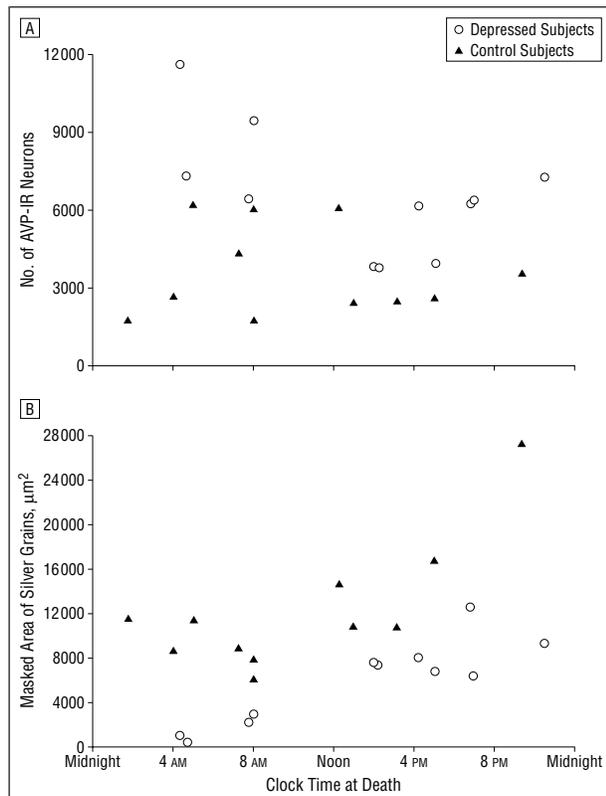


Figure 3. A, Number of vasopressin-immunoreactive (AVP-IR) neurons plotted against clock time at death of each individual (11 depressed subjects, and 11 control subjects). B, Area of masked silver grain plotted against clock time of death of each individual. This figure illustrates that the difference between depressed and control subjects is present at different points of the day and that there is no overlap between the 2 groups when you take the clock time at death into account.

nucleus basalis of Meynert.²⁹ The possibility of a decreased axonal transport rate in depression certainly needs further investigation.

It should be mentioned that the number of cell profiles that expressed AVP-mRNA was higher than the total number of AVP peptide-expressing neurons (controls in this study). This is because, in the ISH study, profiles of cells were counted instead of the number of cells as estimated in the immunocytochemical study. For a comprehensive discussion on the use of the deconvolution or unfolding technique, we refer to a previous study at our institute by Raadsheer et al.³⁰ In this article, a comparison is made between the use of the unfolding method and the dissector and a high correlation was found between both methods ($r_s=0.98$).

Information on the exact influence of antidepressants on the SCN, and, more specifically, AVP in the SCN, is limited. Lithium acts on hamster SCN neuronal firing in vitro, although it is not known on what type of neurons.³¹ It has also been shown that the diurnal rhythm of AVP-mRNA in the rat SCN did not seem to be affected by benzodiazepines.³² Depletion of serotonin in the SCN has been shown to disrupt phase and period characteristics of the daily locomotor rhythm in rats and hamsters.^{33,34} However, the diurnal rhythm of AVP-mRNA of the rat SCN in tissue culture was not disrupted after the administration of the serotonin-depleting agent para-

chlorophenylalanine, a tryptophan hydroxylase inhibitor.³⁴ All these observations argue against treatment effects and support the idea that the alteration of AVP neurons in the SCN might well be related to the trait of depression per se. However, our sample was too small to draw any firm conclusions on the effect of treatment on the outcome measures.

With respect to a possible confounding effect of alcohol on AVP neurons in the SCN in humans, nothing is known. One study by Harding et al³⁵ described that the use of high doses of alcohol is correlated with neuronal degeneration of magnocellular vasopressin neurons in the PVN and SCN. They did, however, not describe an effect on the parvocellular vasopressin neurons in the SCN. In the rat SCN, Madeira et al³⁶ studied the effect of ethanol treatment and withdrawal on AVP-immunoreactivity and mRNA levels in the rat SCN. They found a reduction in the number of AVP neurons in the SCN in both the ethanol-treated and withdrawn rats. Also, the hybridization signal for AVP-mRNA was reduced in both the ethanol-treated and withdrawn rats, with even a weaker signal in the withdrawn rats. This makes it clear that not only the use of alcohol at the time of death should be taken into consideration, but also a possible irreversible effect after alcohol withdrawal during lifetime that could still confound the immunocytochemistry and ISH findings. However, none of the subjects used alcohol at the time of death, as reported by the medical scores. Only 2 subjects, D3 and C3, have a history of alcohol abuse, but these subjects were matched with each other and did not influence our conclusions.

Since the SCN is the clock of the brain, the time of death should also be considered as a possible confounding factor. We excluded this possibility by matching depressed subjects as much as possible with control subjects who had died around the same time (Table). Moreover, a higher number of AVP-IR neurons and a lower amount of AVP-mRNA were found in depressed subjects over the entire period of the day and night (**Figure 3**).

The functional alterations of AVP neurons in the SCN of depressed subjects are of special interest in relation to the impaired regulation of the hypothalamic-pituitary-adrenal system in depression.³⁷ Animal data show that AVP neurons of the SCN exert an inhibitory influence on CRH in the PVN and thereby reduce the stress-induced release of glucocorticoids.¹⁴ Increased levels of circulating glucocorticoids increase AVP-mRNA in the SCN within a narrow time window,³⁸ which will strengthen the inhibition of CRH in the PVN. How exactly the SCN and the hypothalamic-pituitary-adrenal axis are linked to the pathobiology of depression needs further investigation (ie, the feedback mechanism of glucocorticoids on the hypothalamic-pituitary-adrenal axis and on how the SCN is involved in this feedback).

Since this study was performed on postmortem human brain material, antemortem and postmortem factors, such as agonal state, medication, PMD, duration of fixation, and storage time of the tissue, may contribute to the variation observed in mRNA levels.^{39,40} Information on the exact influence of each of these factors on AVP-mRNA levels, however, is still very limited. As far

as PMD is concerned, a significant decrease in the amount of AVP-mRNA, with increasing PMD, was indeed shown in postmortem rat brain.⁴⁰⁻⁴² Relatively few ISH studies on postmortem effects on human brain material have been performed. Using ISH, several human mRNAs have been localized after a PMD of up to 40 hours.^{26,43} In addition, no significant correlation was found between the density of the hybridization signal and PMD (range, 2.5-66 hours) in a comparison of propiomelanocortin mRNA levels in pituitary glands between controls and different diseased patients.⁴⁴ Lucassen et al²⁷ reported that after 6 hours, no further decrease in signal was detected in the AVP-mRNA in the human supraoptic nucleus and paraventricular nucleus of the hypothalamus. In our material, the PMD was 6 hours or longer (Table). We did not find a significant correlation between the number of AVP-IR neurons and PMD or between the amount of AVP-mRNA and PMD in the present study in either the control or in the depressed group, so that there is no indication that PMD might have influenced our conclusions.

CONCLUSIONS

We found an alteration of the AVP neurons in the SCN in depressed subjects, both at the level of AVP-peptide and AVP-gene expression. The results suggest that the functional ability of the SCN to maintain normal biological rhythms is diminished in subjects suffering from depression, which seems to be the result of changes in the balance between production and transport of AVP in the SCN.

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- Meijer JH, Rietveld WJ. Neurophysiology of the suprachiasmatic circadian pacemaker in rodents. *Physiol Rev*. 1989;69:671-707.
- Swaab DF, Van Someren EJ, Zhou JN, Hofman MA. Biological rhythms in the human life cycle and their relationship to functional changes in the suprachiasmatic nucleus. *Prog Brain Res*. 1996;111:349-368.
- Wirz-Justice A. Biological rhythms in mood disorders. In: Bloom FE, Kupfer DJ, eds. *Psychopharmacology: The Fourth Generation of Progress*. 4th ed. New York, NY: Raven Press; 1995:999-1017.
- Souetere E, Salvati E, Belugou JL, Pringuey D, Candito M, Krebs B, Ardisson JL, Darcourt G. Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. *Psychiatry Res*. 1989;28:263-278.
- Van den Hoofdakker RH. Chronobiological theories of nonseasonal affective disorders and their implications for treatment. *J Biol Rhythms*. 1994;9:157-183.
- Rosenthal NE, Sack DA, Gillin JC, Lewy AJ, Goodwin FK, Davenport Y, Mueller PS, Newsome DA, Wehr TA. Seasonal affective disorder: a description of the syndrome and preliminary findings with light therapy. *Arch Gen Psychiatry*. 1984;41:72-80.
- Eastman CI, Young MA, Fogg LF, Liu L, Meaden PM. Bright light treatment of winter depression: a placebo-controlled trial. *Arch Gen Psychiatry*. 1998;55:883-889.
- Yamada N, Martin-Iverson MT, Daimon K, Tsujimoto T, Takahashi S. Clinical and chronobiological effects of light therapy on nonseasonal affective disorders. *Biol Psychiatry*. 1995;37:866-873.
- Moore RY. The organization of the human circadian timing system. In: Swaab DF, Hofman MA, Mirmiran M, Ravid R, Van Leeuwen FJ, eds. *The Human Hypothalamus in Health and Disease*. Vol 93. Amsterdam, the Netherlands: Elsevier Science Publishers; 1992:101-117.
- Raadtsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, Swaab DF. Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. *Neuroendocrinology*. 1994;60:436-444.
- Raadtsheer FC, van Heerikhuizen JJ, Lucassen PJ, Hoogendijk WJ, Tilders FJ, Swaab DF. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. *Am J Psychiatry*. 1995;152:1372-1376.
- Holsboer F, Spengler D, Heuser I. The role of corticotropin-releasing hormone in the pathogenesis of Cushing's disease, anorexia nervosa, alcoholism, affective disorders and dementia. *Prog Brain Res*. 1992;93:385-417.
- Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J Neurosci*. 1994;14:2579-2584.
- Kalsbeek A, Buijs RM, van Heerikhuizen JJ, Arts M, van der Woude TP. Vasopressin-containing neurons of the suprachiasmatic nuclei inhibit corticosterone release. *Brain Res*. 1992;580:62-67.
- American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*. Washington, DC: American Psychiatric Association; 1994.
- Hofman MA, Swaab DF. Diurnal and seasonal rhythms of neuronal activity in the suprachiasmatic nucleus of humans. *J Biol Rhythms*. 1993;8:283-295.
- Hofman MA, Swaab DF. Alterations in circadian rhythmicity of the vasopressin-producing neurons of the human suprachiasmatic nucleus (SCN) with aging. *Brain Res*. 1994;651:134-142.
- Swaab DF, Fliers E, Partiman TS. The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. *Brain Res*. 1985;342:37-44.
- Zhou JN, Hofman MA, Swaab DF. Morphometric analysis of vasopressin and vasoactive intestinal polypeptide neurons in the human suprachiasmatic nucleus: influence of microwave treatment. *Brain Res*. 1996;742:334-338.
- Weibel ER. Stereological methods. In: Weibel ER, ed. *Practical Methods for Biological Morphometry*. Vol 1. London, England: Academic Press Inc; 1979:186-196.
- Goudsmit E, Hofman MA, Fliers E, Swaab DF. The supraoptic and paraventricular nuclei of the human hypothalamus in relation to sex, age and Alzheimer's disease. *Neurobiol Aging*. 1990;11:529-536.
- Cruz-Orive LM. Particle shape-size distributions—the general spheroid problem, II: stochastic model and practical guide. *J Microsc*. 1978;112:153-167.
- Gundersen HJ. Notes on the estimation of the numerical density of arbitrary particles: the edge effect. *J Microsc*. 1977;111:219-223.
- Mohr E, Hillers M, Ivell R, Haulica ID, Richter D. Expression of the vasopressin and oxytocin genes in human hypothalamus. *FEBS Lett*. 1985;193:12-16.
- Mengod G, Charli JL, Palacios JM. The use of in situ hybridization histochem-

- istry for the study of neuropeptide gene expression in the human brain. *Cell Mol Neurobiol.* 1990;10:113-126.
26. Mengod G, Goudsmit E, Palacios JM. In situ hybridization histochemistry in the human hypothalamus. In: Swaab DF, Hofman MA, Mirmiran M, Ravid R, Van Leeuwen FJ, eds. *The Human Hypothalamus in Health and Disease*. Vol 93. Amsterdam, the Netherlands: Elsevier Science Publishers; 1992:45-55.
 27. Lucassen PJ, Goudsmit E, Pool CW, Mengod G, Palacios JM, Raadsheer FC, Guldenaar SE, Swaab DF. In situ hybridization for vasopressin mRNA in the human supraoptic and paraventricular nucleus: quantitative aspects of formalin-fixed paraffin-embedded tissue sections as compared to cryostat sections. *J Neurosci Methods.* 1995;57:221-230.
 28. Salehi A, Lucassen PJ, Pool CW, Gonatas NK, Ravid R, Swaab DF. Decreased neuronal activity in the nucleus basalis of Meynert in Alzheimer's disease as suggested by the size of the Golgi apparatus. *Neuroscience.* 1994;59:871-880.
 29. Salehi A, Verhaagen J, Dijkhuizen PA, Swaab DF. Co-localization of high-affinity neurotrophin receptors in nucleus basalis of Meynert neurons and their differential reduction in Alzheimer's disease. *Neuroscience.* 1996;75:373-387.
 30. Raadsheer FC, Oorschot DE, Verwer RW, Tilders FJ, Swaab DF. Age-related increase in the total number of corticotropin-releasing hormone neurons in the human paraventricular nucleus in controls and Alzheimer's disease: comparison of the disector with an unfolding method. *J Comp Neurol.* 1994;339:447-457.
 31. Mason R, Biello SM. A neurophysiological study of a lithium-sensitive phosphoinositide system in the hamster suprachiasmatic (SCN) biological clock in vitro. *Neurosci Lett.* 1992;144:135-138.
 32. Carter DA, Murphy D. Diurnal rhythm of vasopressin mRNA species in the rat suprachiasmatic nucleus: independence of neuroendocrine modulation and maintenance in explant culture. *Brain Res Mol Brain Res.* 1989;6:233-239.
 33. Morin LP, Blanchard J. Depletion of brain serotonin by 5,7-DHT modifies hamster circadian rhythm response to light. *Brain Res.* 1991;566:173-185.
 34. Honma KI, Watanabe K, Hiroshige T. Effects of parachlorophenylalanine and 5, 6-dihydroxytryptamine on the free-running rhythms of locomotor activity and plasma corticosterone in the rat exposed to continuous light. *Brain Res.* 1979;169:531-544.
 35. Harding AJ, Halliday GM, Ng JL, Harper CG, Kril JJ. Loss of vasopressin-immunoreactive neurons in alcoholics is dose-related and time-dependent. *Neuroscience.* 1996;72:699-708.
 36. Madeira MD, Andrade JP, Lieberman AR, Sousa N, Almeida OF, Paula-Barbosa MM. Chronic alcohol consumption and withdrawal do not induce cell death in the suprachiasmatic nucleus, but lead to irreversible depression of peptide immunoreactivity and mRNA levels. *J Neurosci.* 1997;17:1302-1319.
 37. Deuschle M, Schweiger U, Weber B, Gotthardt U, Koner A, Schmider J, Standhardt H, Lammers CH, Heuser I. Diurnal activity and pulsatility of the hypothalamus-pituitary-adrenal system in male depressed patients and health controls. *J Clin Endocrinol Metab.* 1997;82:234-238.
 38. Larsen PJ, Vrang N, Moller M, Jessop DS, Lightman SL, Chowdrey HS, Mikkelson JD. The diurnal expression of genes encoding vasopressin and vasoactive intestinal peptide within the rat suprachiasmatic nucleus is influenced by circulating glucocorticoids. *Mol Brain Res.* 1994;27:342-346.
 39. Burke WJ, O'Malley KL, Chung HA, Harmon SK, Miller P, Berg L. Effect of pre- and postmortem variables on specific mRNA levels in human brain. *Mol Brain Res.* 1991;11:37-41.
 40. Barton AJ, Pearson RC, Nailerahim A, Harrison PJ. Pre- and postmortem influences on brain RNA. *J Neurochem.* 1993;61:1-11.
 41. Arai H, Noguchi I, Sagi N, Moroji T, Iizuka R. A study of non-isotopic in situ hybridization histochemistry on postmortem changes in vasopressin mRNA in rat brain. *Neurosci Lett.* 1989;103:127-132.
 42. Noguchi I, Arai H, Iizuka R. A study on postmortem stability of vasopressin messenger RNA in rat brain compared with those in total RNA and ribosomal RNA. *J Neural Transm Gen Sect.* 1991;83:171-178.
 43. Solà C, Mengod G, Probst A, Palacios JM. Differential regional and cellular distribution of beta-amyloid precursor protein messenger RNAs containing and lacking the Kunitz protease inhibitor domain in the brain of human, rat and mouse. *Neuroscience.* 1993;53:267-295.
 44. Mengod G, Vivanco MM, Christnacher A, Prost A, Palacios JM. Study on pro-Opiomelanocortin mRNA expression in human postmortem pituitaries. *Mol Brain Res.* 1991;10:129-137.