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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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-IV13 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 microabcesses and infiltration with neutrophilic and eosinophylic 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 immunocytotoxic analysis were replaced by 3 other controls for the in situ hybridization (ISH) study (C2, C7, and C9) (Table).

IMMUNOCYTOCHEMISTRY AND MORPHOMETRY

For immunocytotoxic analysis of AVP, 6-µm-thick paraffin sections through the entire SCN were stained with an antibody against AVP. The immunocytotoxic 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 µm, z-axis). All nuclear profiles within a rectangular grid in one of the oculars that corresponded to 38000 µm² in the section were measured according to Gundersen.21 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

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±8.7 years, for depressed subjects; 70±12 years, for control subjects); brain weight (1280±162.3 g, for depressed subjects; 1399±307.5 g, for control subjects) (P= .96); clock time at death; PMD; and fixation time (38±12.5 hours, for depressed subjects; 34.4±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±SD average PMD (9.5±4.7 hours) than the depression group (32.5±20.4 hours) (Z=-2.67, P=.008), but no significant relationship was found between PMD and the number of AVP-IR neurons (r=0.20, P=.56, for the depressed subjects; r=0.34, P=.31, for the controls) or amount of AVP-mRNA (r=0.15, P=.66, for the depressed subjects; r=0.20, P=.55, for the controls).

The mean±SD of AVP-IR neurons in depression (6589±2389) was found to be significantly higher than in controls (3706±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±3802 µm² vs 12206±5827 µm²) (Z=-2.49;
enough material was left to stain the entire SCN. Hybridization was performed on every 30th 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 438 of the human preprovasopressin precursor. The specificity of the probe has been described previously. 23,24 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 described 27 except for the deproteination and delipidation. Deproteination was done in proteinase-K (10 µg/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 dehydrogenase before hybridization. Each section was incubated with 68-µL hybridization solution containing approximately 1 × 106-cpm-labeled probe. After overnight incubation at 32°C, the sections were rinsed in 1×silver sulfadiazine chlorohexidine for 30 minutes at 50°C, 2 × 30 minutes 0.1×silver sulfadiazine chlorohexidine at 50°C, and 2 × 30 minutes 0.1×silver sulfadiazine chlorohexidine 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 planapla objectives, a blue filter, and a scanning stage. The main principle and procedure of the Interaktive Bild Analyse System measurement have been extensively described before. 28 Briefly, the area of the SCN was manually outlined at low magnification (×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 (×10 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 with the Wilcoxon signed-rank test (2-tailed). Values of P < .05 were considered to be significant. All values are expressed as mean ± 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.

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

P = .01). Furthermore, the mean ± SD area of masked silver grains per profile was significantly lower in depressed subjects (0.33 ± 0.11 µm²) compared with control subjects (0.52 ± 0.15 µm²) (Z = 2.85, P = .004). Although there was a tendency toward a lower mean ± 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 = −0.37, P = .65) or in the amount of AVP-mRNA (Z = −0.95, P = .34) between 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 = .70) or AVP-mRNA (Z = −0.37, 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 = 0.04, P = .89), or for the amount of AVP-mRNA and the duration of the disease (r = 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).
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-

*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.
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 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
nucleus basalis of Meynert.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 al\textsuperscript{35} 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 parvicellular vasopressin neurons in the SCN. In the rat SCN, Madeira et al\textsuperscript{36} 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.\textsuperscript{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.\textsuperscript{14} Increased levels of circulating glucocorticoids increase AVP-mRNA in the SCN within a narrow time window,\textsuperscript{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.\textsuperscript{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 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.20,21 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.


