Hypothalamic functions in patients with pituitary insufficiency
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Citation for published version (APA):

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Arginine vasopressin immunoreactivity is decreased in the hypothalamic suprachiasmatic nucleus of subjects with suprasellar tumors

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ABSTRACT

Suprasellar tumors with compression of the optic chiasm are associated with an impaired sleep-wake rhythm. We hypothesized that this reflects a disorder of the biological clock of the human brain, the suprachiasmatic nucleus (SCN), which is located just above the optic chiasm. In order to test this hypothesis, we investigated the expression of two key neuropeptides of the SCN, i.e. arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP), as assessed by quantitative immunocytochemistry in post-mortem hypothalamic tissue of patients with a suprasellar tumor inducing permanent visual field defects.

Post-mortem hypothalamic tissue of five patients with a suprasellar tumor inducing permanent visual field defects (acromegaly n=2, nonfunctioning macroadenoma n=1, macroprolactinoma n=1, infundibular metastasis of a colorectal adenocarcinoma n=1) and fifteen age- and gender-matched controls was obtained from the Netherlands Brain Bank. Total AVP-immunoreactivity in the SCN was lower in patients with a suprasellar tumor than in controls \((p = 0.03)\). By contrast, total VIP-immunoreactivity was not different between patients and controls \((p = 0.44)\). Suprasellar tumors leading to permanent visual field defects are associated with reduced AVP-, but not VIP-immunoreactivity in the SCN. These findings raise the possibility that selective impairment of the SCN contributes to sleep-wake disturbances in these patients.
INTRODUCTION

Patients previously treated for a suprasellar tumor often experience subjective sleep impairment. Recent studies reported altered sleep characteristics despite proper endocrine substitution therapy for pituitary insufficiency (2;16;19;22). The underlying mechanisms are incompletely understood at present, but alterations in the suprachiasmatic nucleus (SCN), which is the biological clock of the brain, are presumed to be a key factor.

Compression of the optic chiasm by a suprasellar tumor causes visual field defects (7), reflecting mechanical impairment of this anatomical structure. These tumors may cause injury to adjoining tissues as well, including the mediobasal hypothalamus containing the SCN. This hypothalamic nucleus is located immediately above the optic chiasm, and is responsible for maintaining circadian rhythms such as sleep/wakefulness (18). Recently, we demonstrated that compression of the optic chiasm due to a tumor with suprasellar extension is associated with permanent shorter sleep duration (4), supporting the concept that expanding tumors in the sellar region may harm the SCN, which in turn leads to sleep disturbances.

We were in the unique position to study a small group of deceased subjects (n=5) with a diagnosis of a suprasellar tumor inducing visual field defects. In these subjects and in matched control subjects without a suprasellar tumor, we investigated the expression of two key neuropeptides of the SCN, i.e. arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP), as assessed by quantitative immunocytochemistry in post-mortem hypothalamic tissue.

MATERIAL AND METHODS

Subjects

Post-mortem hypothalamic tissue of 5 patients and 15 control subjects was studied; for each patient, 3 age- and gender-matched control subjects were included. All patients had established visual field defects in the presence of a suprasellar tumor according to their medical records. The tumor was resected in three patients (subjects #5, #13 and #17) and postoperative radiotherapy was administered to one patient (subject #17). Pituitary insufficiency was present in three patients (subjects #1, #13 and #17) and they received hormonal substitution. Data on surgery, radiotherapy and pituitary insufficiency was not available for respectively one (subject #9), three (subjects #5, #9 and #13) and one (subject #5) patient(s). Clinicopathological data are described in table 1.

All brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience (director Dr. I. Huitinga) in accordance with the formal permissions for
brain autopsy and for the use of human brain material and clinical information for research purposes.

**Histology**

Brains were dissected at autopsy and the hypothalamus was fixed in 10% phosphate-buffered formalin at room temperature for 1-32 months. After dehydration in graded ethanol series, tissues were cleared in xylene and embedded in paraffin. Coronal serial sections (6mm) were cut over the entire rostro-caudal axis. For anatomical orientation, every 100th section was collected and mounted on chrome alum-gelatin coated glass slides or Superfrost plus slides (Menzel Glaser, Germany) and subsequently dried for two days at 37°C, followed by Nissl staining. Two series of consecutive sections were mounted at 50-section intervals over the entire rostro-caudal axis of the SCN, which were used for AVP- and VIP- immunocytochemistry. To ensure sampling over the entire rostro-caudal axis of the SCN we made sure that at least one section negative for AVP- and VIP staining was included at the rostral as well as the caudal border of the nucleus.

**Antibodies**

We used polyclonal rabbit antisera raised against synthetic peptides derived from human AVP (Truus, 29-01-89, Netherlands Institute for Neuroscience) and human VIP (Viper, 18-09-1986, Netherlands Institute for Neuroscience) for immunocytochemical staining. Antiserum from the final bleed was used without further purification. Antibody specificity has been described previously (6;8;23).

**Immunocytochemical procedures**

Sections were mounted on Superfrost plus slides and dried for at least 2 days at 37°C. After deparaffinization in xylene and rehydration through graded ethanol series, sections were washed in TBS and antigen retrieval was performed using microwave treatment (10 min, 700W) in TBS at pH 7.6. After cooling down to room temperature (RT), sections were incubated in the primary antibody diluted 1:800 in SUMI [supermix: 0.05M Tris, 0.15M NaCl, 0.5% Triton X-100 (Sigma, Zwijndrecht, The Netherlands), and 0.25% gelatin (Merck Darmstadt, Germany) (pH 7.6)] for AVP and 1:5000 for VIP overnight at 4°C in a humidified chamber. Sections were washed in TBS and incubated in the secondary antibody (biotinylated goat anti-rabbit, 1:400 in SUMI) for 1h at RT. After washing in TBS, sections were incubated for 1h at RT in avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA) and subsequently rinsed in TBS. Finally, staining was visualized using 0.5 mg/ml 3,3'-diaminobenzidine (Sigma) in TBS containing 0.2% ammonium nickel sulfate (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H$_2$O$_2$ (Merck) for approximately 15 min. The reaction was stopped in distilled water. The sections were dehydrated in graded ethanol series, cleared in xylene, and coverslipped using Entellan (Merck).
Quantitative analysis

For quantification of the immunoreactive signal, gray values of the DAB-Ni precipitate in the SCN were analyzed by computer-assisted densitometry using Image pro (Media Cybernetics, Silver Spring) and software developed at the Netherlands Institute for Neuroscience. Every 50th section containing the SCN was analyzed. The SCN was manually outlined by an investigator blind to the diagnosis of the studied subjects (AA). Estimates of the total amount of immunoreactivity were made according to Cavalieri’s principle based on the multiplication of the masking volume of the DAB-Ni precipitate by the sampling frequency, resulting in arbitrary units (a.u.) representing μm³ multiplied by staining intensity as determined by our image analysis system (1).

Statistical analysis

To normalize their skewed distribution, the AVP- and VIP-immunoreactivity a.u.’s were log-transformed prior to statistical analysis. Mixed effect regression models (MLwiN, Centre for Multilevel Modeling, Institute of Education, London, UK) were used to estimate the group differences while accounting for the data structure of three matched controls for each patient. The effects on AVP- and VIP-immunoreactivity of post-mortem delay, fixation time, clock time of death and month of death were evaluated by including them as regressors in the model. z-Tests were used to obtain the significance of the effect sizes, where z is the ratio of the estimated difference over the standard error of its estimate (21). Two-sided p-values below 0.05 were considered significant.

RESULTS

In subject #1 the tumor mass invading the hypothalamus was clearly visible. In subjects #5, #9, #13 and #17 no gross neuroanatomical abnormalities were observed. AVP- and VIP-staining was observed in the SCN of all subjects. Staining intensity showed a considerable inter-individual variation in patients as well as in controls. The log-transformed SCN AVP-immunoreactivity was 18 ± 7% lower in patients with a suprasellar tumor than in the control group (p = 0.03) (figure 1A). Staining is illustrated in figures 1B and 1C. By contrast, total VIP-immunoreactivity was not different between patients and controls (p = 0.44) (figures 1D-F). Comparison of staining intensity, and differences in the area masking the signal as well as the number of objects that were stained did not yield any significant results. There was no significant effect of post-mortem delay, fixation time, clock time of death or month of death on AVP-immunoreactivity (0.06 < p < 0.98, average p = 0.56) or VIP-immunoreactivity (0.07 < p < 0.78, average p = 0.56).
Chapter 6

DISCUSSION

In the present study we demonstrate for the first time a reduction of total AVP- but not VIP-immunoreactivity in the SCN of patients with a suprasellar tumor inducing visual field defects. These results provide immunocytochemical support for functional changes in the SCN in these patients. Comparison of staining intensity and number of objects stained showed no differences between our experimental groups. It therefore seems that neither a decrease in protein expression per neuron, nor a decrease in the number of neurons as estimated by the number of stained objects can fully explain the decrease in AVP-expression. It is feasible that the observed difference in total AVP-immunoreactivity is caused by a combination of factors amplifying each other.

One could speculate that the observed difference in total AVP-immunoreactivity may have been affected by seasonal and diurnal variations (9). Unfortunately, sleep characteristics were only available for subject #1. However, we did not find any statistical correlation between total AVP-immunoreactivity and clock time or month of death. Moreover, diurnal oscillations have been described only in young subjects, and were found to be blunted in subjects over 50 years of age (9). Since our study population consisted of older subjects it

FIGURE 1. Graphical representation of difference in total AVP- (A) and VIP-immunoreactivity (D) in the SCN between patients and controls. Each dot represents one subject. B&C: Representative coronal sections of the SCN in a patient (#13) and control (#14) person stained for AVP. E&F: Representative coronal sections of the SCN in a patient (#13) and control (#14) person stained for VIP. Inserts represent high power magnifications.

Note that total AVP, but not VIP-immunoreactivity was lower in patients than controls. Statistical analysis was performed using mixed effect regression models and z-tests. Scale bar for B, C, E, F = 100μm. Asterisks indicate the third ventricle.
AVP in SCN of subjects with suprasellar tumors

is therefore unlikely that diurnal or seasonal variations influenced our results. Although all brain material is processed according to standardized protocols, variation in post-mortem delay and fixation time cannot be excluded. However, correlation analyses of these

<table>
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<th>Subject</th>
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<th>Age</th>
<th>PMD</th>
<th>Fix</th>
<th>CTD</th>
<th>MoD</th>
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CTD clock time of death; F Female; Fix Fixation time in days; M male; MoD month of death; nd not determined; NFMA non-functioning macroadenoma; PMD postmortem delay in hours.
potential confounders on total AVP- and VIP-immunoreactivity did not yield any significant results. Interestingly, we found a decrease in AVP-, but not VIP-immunoreactivity. AVP and VIP are the two main neuropeptide components of SCN neurons, expressed in two distinct neuronal subpopulations of the SCN (17). Both are highly important in circadian timekeeping as evident from animal experimental studies. The SCN generates a circadian rhythm that is entrained by the daily light-dark changes of the environment (3;14). VIP expressing neurons serve as the main targets of retinal input to the SCN and play a critical role in transmitting the light effect to the SCN, whereas AVP neurons amplify the amplitude of the circadian rhythm (10;13) and translate this circadian signal to target areas (11). Why we only observed decreased AVP-, but not VIP-immunoreactivity remains unclear, but it may reflect differential susceptibility of these neuronal populations to mechanical stress.

Human brain material of subjects who had experienced permanent visual field defects as a result of a suprasellar tumor is not readily available. However, we managed to include 5 patients in our studies and matched each of them with 3 age- and gender-matched controls. One patient (subject #5) suffered from a very rare condition known as hereditary cerebral hemorrhage amyloidosis in addition to the suprasellar tumor. Hereditary cerebral hemorrhage amyloidosis is an inherited disease characterized by amyloid deposits in cerebral blood vessels, resulting in (recurrent) hemorrhagic strokes and dementia (5). Since we were unable to match this subject with other subjects with the same type of neurodegenerative disease, we matched him with two Alzheimer patients, to control at least for neurodegenerative disease, in addition to one control subject with cerebral hemorrhage. Of note, this subject #5 showed a similar expression level of AVP and VIP compared with the other patients.

Whether the observed changes in the SCN contributes to the development of sleep disturbances in patients with expanding suprasellar tumors remains an intriguing question. Due to the retrospective and anatomical character of this study, we have no functional data on sleep characteristics in these subjects, except for one patient (subject #1). This subject was reported to experience not only blurring of vision in the right temporal field, but also to require more sleep at night (20). This information, together with previous studies reporting excessive daytime sleepiness and altered sleep characteristics, indicates that the cases we studied would likely have suffered from circadian sleep disturbances (1-4,7). Additionally, a recent report showed that the SCN in rats is not only involved in circadian regulation of the sleep-wakefulness cycle (15) but that subregions of the SCN can regulate sleep stages as well (12). Therefore, it is feasible that the reduction in AVP-immunoreactivity found in our human study contributes to sleep disturbances previously observed in patients with suprasellar tumors, although this is highly speculative at this stage. It is feasible that mechanical damage as a result of long-term compression could cause reactive gliosis and microglial activation. However, in three of the five subjects studied here the tumor was resected and compression was relieved. Therefore, we are not
confident that studies on gliosis and microglial activation in this group of subjects would yield any reliable results.

In summary, despite the predominantly descriptive character of the study and the relatively low number of observations, we do find support for SCN changes in patients with a suprasellar tumor that induce permanent visual field defects. Therefore, we feel we provide valuable new data relevant for understanding the pathogenesis of sleep disturbances in these patients. An interesting possibility is that the reduction in total AVP-immunoreactivity found in this study contributes to sleep disturbances commonly seen in patients with suprasellar tumors.

**ACKNOWLEDGEMENTS**

Brain material was obtained from the Netherlands Brain Bank (Director I. Huitinga). We wish to acknowledge Bart Fisser for his excellent technical assistance.
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