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Research report

Estimation of oxytocin mRNA in the human paraventricular nucleus in AIDS by means of quantitative in situ hybridization

Stephan E.F. Guldenaar^{*}, Dick F. Swaab

Graduate School of Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

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Abstract

Recently, a 40% reduction in the total number of oxytocin immunoreactive neurons of the paraventricular nucleus of the hypothalamus in AIDS patients was reported. In the present study, we determined whether this decrease is associated with a diminished amount of oxytocin mRNA. We used in situ hybridization combined with densitometric image analysis for the quantitative assessment of oxytocin gene expression in the paraventricular nucleus of a group of AIDS patients ($n = 10$) and a carefully matched control group ($n = 8$). We found no significant difference ($P = 0.08$) in the amount of oxytocin mRNA per total paraventricular nucleus between the two groups. In addition, no significant differences were found in the part of the volume of the paraventricular nucleus that was occupied by hybridized cells ($P = 0.12$) or in the mean signal density ($P = 0.08$). The findings do not support the hypothesis that the extensive decrease in oxytocin immunoreactive neurons of the paraventricular nucleus in AIDS is associated with a decrease in total oxytocin mRNA content in this nucleus. The data are compatible with the suggestion that in AIDS the biosynthesis of oxytocin is changed in an unknown way at the (post)transcriptional level.

Keywords: Oxytocin; AIDS; Paraventricular nucleus; In situ hybridization; Human brain; Immunocytochemistry

1. Introduction

The paraventricular nucleus of the hypothalamus (PVN) contains a number of neuropeptides with functions in the central nervous system as well as in the periphery. By virtue of these peptides the PVN may play a role in autonomic and endocrine dysfunction in AIDS.

Oxytocin (OT) is a neuropeptide that is synthesized in the PVN as part of a larger precursor molecule from which it must be split off and processed before it can exert its functions [13]. OT plays a role in lactation [12], reproduction [4,14] and steroidogenesis [24]. It is of interest that changes in the hypothalamo-pituitary-gonadal axis [8,20] and hypogonadism [9,17] have been described in AIDS. Furthermore, OT may be relevant in AIDS because it is implicated in cognition [3] and immunomodulation [11,23]. While a role of OT in the pathogenesis of AIDS has not been shown, a 40% reduction in the total number of OT immunoreactive (OT-IR) cell bodies in the PVN [25] represented neuroanatomical evidence supporting the in-

volvement of OT cells of the human PVN in AIDS. The cause of this reduction has not been studied. We determined whether the decrease in the number of OT-IR cells in the PVN is associated with a diminished amount of OT mRNA which would suggest a downregulation of the OT gene in the PVN in AIDS. Gene expression of neuropeptides in human postmortem brain tissue can be studied by means of in situ hybridization [18,19,27,29]. In the present study, we used quantitative in situ hybridization to determine total OT mRNA in the PVN of AIDS- and control patients.

2. Materials and methods

2.1. Tissues

Brains of 10 subjects (8 male and 2 female) who had died of AIDS and 8 controls (7 male and 1 female) who had died of other causes, but had not suffered from primary neurological or psychiatric disorders, were obtained at autopsy. The brains were weighed and the hypothalami dissected. Hypothalami were fixed in 10% for-

^{*} Corresponding author. Fax: (31) (20) 6961006.

malin at room temperature and subsequently dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin.

Neuropathology was performed either by Dr. W. Kamphorst (Free University of Amsterdam) or Dr. D. Troost (Academic Medical Center of the University of Amsterdam). AIDS patients were diagnosed according to the criteria of the Centers for Disease Control [7]. Clinical diagnoses are presented in Table 1. The sexual orientation of the AIDS but not the control patients was identified. Seven AIDS patients were male homosexuals; of the remaining 3 heterosexuals, 2 were female and 1 male. The distinction based on sexual orientation was made because in the suprachiasmatic nucleus from the same patients a significant difference was found in the number of neurons that were stained immunocytochemically for vasopressin, a peptide closely related to OT [30]. The subjects in each group were selected in such manner that similarity of mean and standard deviation values between both groups were obtained for the following variables: age of the subjects, the postmortem interval (PMI), fixation- and storage time of the tissues (Table 1). Thionin stained serial sections with a thickness of 6 μm and a section-to-section interval of 300 μm were used to locate the PVN histologically. In addition, sections previously immunostained for OT were used to verify immunocytochemically the PVN boundaries.

2.2. *In situ* hybridization

Duplicate or triplicate section series of each patient were collected. Each series contained between 9 to 14 sections (mean $\pm$ S.D.: 12 ± 1.5). From both the AIDS and control group, 5 extra series were used for the estimation of the interassay variation which was inherent to the use of more than one assay needed to process the large number of sections. Patients from each group were divided randomly over 3 hybridization assays. After random selection of a first section of each series within the histologically and immunocytochemically defined range of the PVN, the other sections of each series were collected with a sampling interval of 600 μm . The 2 outermost sections were taken from beyond the defined rostrocaudal range of the PVN in an attempt to obtain zero points for the analysis. Sections were mounted with sterile water on 2% amino-alkyl-silane (Sigma) coated microscope slides. Tissues were dried for 48 h in a stove at 38°C and subsequently kept at room temperature until pretreatment and hybridization.

The sections were deparaffinized in xylene and brought to phosphate buffered saline (PBS) via a descending series of ethanols. The sections were treated with 0.2 N HCl for 20 min [18,19] and, after 2 washes in PBS of 5 min each, deproteinized with proteinase K. In pilot experiments, an optimal hybridization signal was found with a concentration of 10 μg proteinase K/ml buffer (10 mM Tris, 2 mM CaCl_2) at 37°C for 15 min. However, since protease activity may differ between batches optimization may be

required. The protease treatment was stopped by a rinse in glycine buffer (2 mg/ml PBS) followed by two rinses in PBS. Subsequently the sections were dehydrated in an ascending ethanol series, delipidated with chloroform for 5 min [18,27] and dipped in 100% ethanol before drying in a vacuum desiccator where they were kept for 1 to 3 days until the start of the *in situ* hybridization incubation.

The OT probe, a 45-mer oligonucleotide complementary to bases 1166–1210 of the human prepro-oxytocin-neurophysin I gene (GenBank Accession No.: M11186) was a gift from Drs. G. Mengod and J.M. Palacios (Laboratory Almirall, Barcelona). The probe was made on a 380B Applied Biosystems DNA synthesizer and purified by electrophoresis on a 20% polyacrylamide/8 M urea gel. No interfering homologies with other known sequences in established databases were found using BLAST [1].

The OT probe was 3'-labelled in 2 reactions using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α - ^{35}S]dATP (> 1400 Ci/mmol, Dupont-NEN) to a specific activity of 12,000 Ci/mmol probe and was recovered by ethanol precipitation. The pellet was resuspended in 100% formamide.

The labelled OT probe was mixed and diluted in hybridization buffer (HBF) (0.5M NaCl, 1 $\times$ Denhardt's, 10 mM Tris (pH 7.6), 1 mM EDTA, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, 50% formamide, 50 mM DTT) at a previously established saturating concentration of 0.86 pmol/ml HBF (J. UdodeHaes and S.E.F. Guldenaar, unpublished results).

Hybridizations took place on 3 consecutive days. The sections were coverslipped and hybridized overnight in a humidified stove set at 33°C. The following day the coverslips were gently removed in 2 $\times$ standard saline citrate (SSC; 1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and the sections were washed sequentially in: 1 $\times$ SSC for 15 min at room temperature, 2 $\times$ in 0.1 $\times$ SSC for 30 min at 45°C, 2 $\times$ in 0.1 $\times$ SSC for 30 min at room temperature. Subsequently, the sections were dehydrated in 300 mM ammonium acetate (pH 5.5)/absolute ethanol at volume ratios of 1:1, 3:7, 1:9, 0:1 respectively and dried in a stream of cool air. The sections were apposed directly to autoradiography film (Hyperfilm- β max, Amersham) and exposed for 24 h. Films were developed for 4 min in Kodak D-19 at 20°C and fixed in Kodak Unifix for 8 min.

Specificity of the OT probe in this protocol was evaluated using a sense probe (Perkin Elmer), by displacement with unlabelled OT probe and comparison with the established immunocytochemical localization of OT cells.

2.3. Quantitative analysis

Grey values of the film autoradiograms were analysed by computer-assisted densitometry using an IBAS image analysis system (Kontron) and software developed at our institute by Dr. C.W. Pool. The relationship between the

Table 1
Patient data of AIDS and control group

Patient Nr.	Sex	Age (years)	PMI (h)	FTime (days)	STime (months)	Brain weight (g)	Clinical diagnosis	Treatment	
								AZT	AF
8979.3	M	25	47	28	64	1530	AIDS, pneumonia, acute bronchitis	0	0
89116	M	30	8	26	63	1340	AIDS, disseminated non-Hodgkin lymphoma, intravenous drug abuse	+	+
86426	M	32	49	11	91	1440	AIDS, bilateral bronchopneumonia, anaemia, cerebral toxoplasmosis	0	0
88309	F	34	12	24	70	1400	AIDS, disseminated histoplasmosis	0	+
87396	M	39	24	28	80	> 1330 *	AIDS, progressive multifocal leucoencephalopathy	+	0
882850	M	41	12	34	71	> 1240	AIDS, viral encephalitis, cerebral toxoplasmosis, cytomegalic virus, bronchopneumonia	+	+
883845	M	42	19	30	68	1340	AIDS, meningencephalitis, cytomegalic infection, aspergillus abcess in cisterna magna	+	+
8722.4	M	43	24	17	90	> 1340	AIDS, disseminated Kaposi sarcoma	0	+
86381	M	62	ND	19	92	1350	AIDS, AIDS dementia complex, Kaposi sarcoma	0	+
89224	F	73	48	38	60	> 1090	AIDS, pneumonia, epilepsy	ND	ND
mean		42.1	27	25.5	74.9	1400			
S.D.		14.8	16.6	8.2	12.3	75			
C.V.		0.35	0.61	0.32	0.16	0.05			
88140.5	M	23	48	34	75	1540	Transmural myocardial infarct	ND	ND
87110	M	23	13	11	87	1310	Brainstem encephalitis	ND	ND
8944.1	M	28	ND	29	65	1545	Cardiac arrest	ND	ND
9090.1	M	30	5	ND	52	1325	Tetralogy of Fallot	ND	ND
87260	M	37	48	46	72	1510	Alcohol intoxication combined with benzodiazepines	ND	ND
8839.1	M	41	ND	33	78	1500	Fortral and benzodiazepine intoxication leading to respiratory depression and heart failure	ND	ND
89411	F	49	24	31	55	1260	Metastasized lung carcinoma, septicaemia, acute respiratory distress syndrome	ND	ND
90762	M	64	13	48	53	1410	Cardiac infarction, lung emphysema	ND	ND
mean		36.9	25.2	33.1	67.1	1425			
S.D.		14.2	18.7	12.2	13	114			
C.V.		0.38	0.74	0.37	0.19	0.08			

AF, antifungicide; AZT, zidovudine; C.V., coefficient of variation; F, female; FT, fixation time; M, male; ND, not determined; PMI, post mortem interval; S.D., standard deviation; STime, storage time; 0, untreated; +, treated.

* > 1330 means that a piece with unknown weight was removed. The rest of the brain weighed 1330 g.

grey values and the amount of radioactivity was assessed with radioactive standards. A standard range of 10 values from 100 cpm/ μ l to 25,600 cpm/ μ l was obtained by serial dilution of the labelled probe in HBF. 1 μ l samples of each standard were spotted on filter paper (Whatman 41) and co-exposed with the hybridized sections. For each section series the profile of integrated density values through the entire PVN was determined using procedures that we reported [18]. In brief, the labelled area of the PVN and the structure weighted, background corrected mean density of each section were determined and used to estimate the total amount of radioactive label present in the PVN. This was used as a relative measure for the amount of OT mRNA detected in the PVN tissue. Integration of the area values was used to estimate the volume of the PVN occupied by hybridized cells i.e. Integrated Area (IA) and the mean of the integrated densities (MID) of each PVN was calculated.

2.4. Statistics

A two tailed Mann–Whitney *U*-test was used for comparisons between the AIDS and control group. Relations

between the total amount of radioactive label in the PVN and matching variables (age, PMI, fixation time and storage time) were determined using linear regression. Results were considered statistically significant if $P < 0.05$.

3. Results

The OT probe hybridized to cells of the PVN and the supraoptic nucleus (SON) (Fig. 1a). In the SON, hybridized cells were found in the dorsal part which is an established preferential site of OT neurons in this nucleus (Fig. 1a). This preferential localization supported the specificity of the OT probe. The specificity of the labelled OT probe was also evidenced by the absence of specific hybridization signal by the labelled OT sense probe (Fig. 1b). Furthermore, specificity of the OT probe was validated by the finding that the hybridization signal (Fig. 2a) was displaced by excess unlabelled anti-sense probe in a concentration dependent manner using 5-, 10-, 20- and 50-times excess of unlabelled probe. Displacement appeared to be complete at 50-fold excess of unlabelled probe (Fig. 2b).

Fig. 1. Adjacent sections through the hypothalamus of an AIDS patient (#89793) demonstrate the hybridization of the oxytocin-probe in the human paraventricular nucleus (pvn) (a). The specificity of the hybridization of the oxytocin probe was supported by the absence of signal when a sense probe was used on an adjacent section (b) and by the hybridization in the dorsal cap of the supraoptic nucleus (son) (a) which is in agreement with the established, preferential localization of oxytocin cells in this nucleus. III, third ventricle; oc, optic chiasm. Bar = 5 mm

From all patients, samples were obtained along the rostro-caudal axis to identify the rostral and caudal end of the PVN. In 85% of the patients the first and/or last sampled section still contained a small number of hybridized cells that, judged by their location, belong to the PVN, whereas the PVN was expected to be absent in these sections of each sample range based on the lack of OT immunostained PVN cells and thionin histology. The estimated length of the PVN along the rostro-caudal axis of AIDS- and control patients was $5880 \pm 882 \mu\text{m}$ and $6075 \pm 1140 \mu\text{m}$, respectively (mean $\pm$ S.D.) and not significantly different ($P = 0.75$).

No significant differences in amount of OT mRNA per PVN ($P = 0.8$), MID values ($P = 0.2$) or the IA ($P = 0.4$) of the PVN related to sexual orientation were found within the AIDS group. Accordingly, no distinction based on homo- or heterosexuality was made in the further analysis of the data.

No significant difference was found in the amount of OT mRNA in the PVN between the AIDS- and the control group ($P = 0.08$; Fig. 3a). Similarly, no significant differences were found in the MID values ($P = 0.08$; Fig. 3b) or in the integrated area of the PVN between the two groups ($P = 0.12$; Fig. 3c).

In the AIDS group no correlation between the amount of OT mRNA and either age ($P = 0.53$), PMI ($P = 0.42$) or fixation time ($P = 0.10$) was found. Storage time yielded, however, a significant correlation with a higher amount of OT mRNA found in older tissue ($P = 0.02$). The significance depended heavily on a single datapoint that constituted the highest OT mRNA amount in the longest stored tissue of this group. After omission of this datapoint and re-analysis of the data by linear regression, only a non-significant trend was found ($P = 0.06$), so that we cannot attribute any meaning to this correlation. In the control group no significant correlation of any of the matching variables with the level of OT mRNA in the PVN was found: $P_{\text{age}} = 0.10$; $P_{\text{PMI}} = 0.61$; $P_{\text{fixation time}} = 0.053$; $P_{\text{storage time}} = 0.78$.

No relation was found between AZT and/or antifungal treatment and the amount of OT mRNA.

The interassay variation in the observations due to dividing the sections over 3 different in situ hybridization assays was $20.9 \pm 5.8\%$ (mean $\pm$ S.E.M.). The variation of the detected amount of OT mRNA within the AIDS and control group was 23.2% and 42.2% respectively. The variation of the IA and MID within the two groups was 10.7% resp. 26.4% and 15.7% resp. 28.0%

Fig. 2. Adjacent sections through the hemi-hypothalamus of an AIDS patient (No. 87224) demonstrate that the signal obtained with the labelled oxytocin probe in the paraventricular nucleus (pvn) (a), was displaced by a 50-fold excess of unlabelled probe (b). Bar = 2 mm

4. Discussion

The presence and distribution of OT mRNA containing cells in the human PVN of the hypothalamus has been demonstrated previously in a qualitative manner [19,29]. Our study is consistent with these reports. In addition, our quantitative data demonstrated the degree of variation in OT mRNA between samples of human PVN. Besides its biological component, variation in the amount of mRNA in human brain material has been attributed to a number of ante- and postmortem variables that include the ones we used for the selection of our patients [5,6], but we found no significant correlations. Recently, a correlation between tissue pH and preservation of several mRNAs, but not OT mRNA, was shown in human postmortem brain [15]. We have no records of the brain tissue pH of our patients and cannot exclude the possibility that this variable would be correlated with the presented OT mRNA findings. The interassay variation due to the different hybridizations ($20.9 \pm 5.8\%$ (mean $\pm$ S.E.M.)) compared well with the experiment-to-experiment variation in *in situ* hybridization experiments in the hypothalamus of the rat (McCabe, personal communication).

Analogous to the PVN of the rat [32] and hamster [21], the human PVN is suggested to contain two functionally-distinct populations of OT neurons; one with a neuroendocrine role and one with central functions. In the human PVN, however, these two OT cell types cannot be distinguished clearly because, unlike in rat and hamster, there appears to be neither a discrete size distribution nor a regional separation of the two populations [31]. Furthermore, the cells cannot be defined by their projections without the ability to apply tracing techniques in the human brain. Since it is not possible to determine with certainty to which population an OT neuron of the human PVN belongs, we chose not to study OT mRNA expression at the level of the cell but rather to quantify the total amount of OT mRNA per PVN.

We did not apply *in situ* hybridization and immunocytochemistry on the same section and hence we cannot exclude the possibility of a discrepancy in the detection of OT cells between both techniques. Furthermore, such discrepancy between our findings and the immunocytochemical data by Purba et al. [25] will be confounded by a difference in sensitivity of both techniques. However, we found an *in situ* hybridization signal in 85% of all the sections that were either the first or last of the series'

Fig. 3. No significant difference was found in the total amount of oxytocin mRNA in the paraventricular nucleus (PVN) between AIDS- and control patients (a) ($P = 0.08$). Likewise, no differences were found in the estimated volume of the PVN occupied by hybridized cells (b) ($P = 0.12$) or in the mean integrated density of the hybridization signal (c) ($P = 0.08$). The data are presented as individual datapoints and as mean $\pm$ standard deviation. AU, arbitrary units

ranges whereas these sections were indicated to be devoid of OT cells by thionin staining and immunocytochemistry [25]. This indicates that our OT mRNA in situ hybridization method, if not more sensitive, is at least as sensitive as the immunocytochemical technique by Purba et al. [25].

In the present study, we found no significant differences in the total amount of detectable OT mRNA in the PVN between AIDS- and control patients. Thus, our findings do not support the suggestion that the decrease in OT-IR cell numbers of the PVN in AIDS would be associated with a decrease in OT mRNA in this nucleus.

Several explanations for the apparent discrepancy between our current in situ hybridization findings and the immunocytochemical data by Purba et al. [25] may be provided, including (1) differences in OT mRNA abundance between centrally projecting- and neuroendocrine OT cells, (2) defective OT gene transcripts, and (3) differences at the posttranscriptional level between OT neurons of AIDS and control patients.

With respect to the first possibility it should be noted that our method estimates the total amount of OT mRNA in the PVN and depends on the mRNA copy number per cell. While differences in the latter could be related to a general heterogeneity among OT cells, it is widely held that centrally projecting OT neurons contain less OT mRNA than their endocrine counterparts. However, at present it is not possible to quantify such difference in man since the distinction between the two cell populations cannot be made with certainty as we explained earlier. The current findings may be explained by suggesting that most of the amount of OT mRNA in the PVN is contributed by neuroendocrine cells that project to the neural lobe of the pituitary and that the 40% decrease in the OT-IR cell population [25] is related to the inability to stain for OT in centrally projecting PVN neurons in AIDS. If this is true, then the decrease of OT-IR cells in AIDS would be more likely related to OT functions in the central nervous system rather than to the neuroendocrine role of OT.

The second possibility that OT cannot be produced in PVN neurons of AIDS patients because its gene transcript is defective should also be considered [22]. To date, no studies have addressed the occurrence and consequences of changes in transcriptional processes of the OT gene in AIDS.

Regarding the third possibility, discrepancies between in situ hybridization and immunocytochemistry related to posttranscriptional events have been described in other systems. For instance, in a comparative study of insulin and pre-pro-insulin mRNA in streptozotocin-induced diabetic rats and normoglycemic animals, Van Gompel et al. [33] showed that, whereas in normoglycemic rats the ratio between cells identified by immunocytochemistry and in situ hybridization was > 0.95 , in diabetic rats this ratio was < 0.20 . Their data constitute compelling evidence for the suggestion that posttranscriptional events become critical determinants in the production of insulin during a state

of induced diabetes and, in general terms, may affect peptide biosynthesis during disease.

In addition, Altstein and Gainer [2] found that during fetal development a selective delay in the expression of a processing enzyme in OT hypothalamic neurons lead to a discrepancy between the detection of fully processed OT and its precursor from embryonic day 17 to 21. The data implied that the OT gene expression in the hypothalamus of the rat started about 4 days before the first detection of mature OT and OT transcripts have indeed been detected on embryonic day 17–18 [16]. Similarly, OT mRNA and OT precursor are detected earlier than fully processed OT in granulosa cells during the development from preovulatory follicle to corpus luteum [10,34]. Furthermore, this mechanism may play a role in the decrease of OT in extrahypothalamic brain areas of the aged rat [28]. It should also be mentioned that the presence of some inactive, altered and modified proteins, e.g. enzymes, is associated with age- and disease-related posttranslational modifications [26]. Thus, our data would be compatible with the suggestion that in AIDS the relation between fully processed OT and its mRNA is modified by different OT precursor processing. Impaired OT production and release have direct consequences for the neuroendocrine role of OT in labour and lactation [14]. However, in women with AIDS these roles of OT have not been studied. In men with AIDS a high prevalence of impotence and decreased libido have been found [8,9,17,20]. A role of OT in these problems is conceivable, since there is good evidence that OT affects male reproduction as a neurohypophyseal hormone and as a centrally active substance [4,14].

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