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

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Received 15 April 1994; revised 8 August 1994; accepted 18 August 1994

Abstract

In order to study the suitability of formalin-fixed paraffin-embedded brain tissue for vasopressin (AVP)-mRNA detection, we used symmetric halves of 5 human hypothalami. In every case, one half was formalin fixed for 10–35 days and paraffin embedded while the other half was frozen rapidly. Following in situ hybridization (ISH) histochemistry on systematically obtained sections of the supraoptic (SON) and paraventricular nucleus (PVN) of both halves, total amounts of AVP-mRNA in these nuclei were estimated using densitometry of film autoradiographs. Total amounts of radioactivity were found to vary considerably between patients and amounted to 1297 ± 302 arbitrary units (AU) (PVN) (mean $\pm$ SEM) and 2539 ± 346 (SON) for the cryostat sections and 868 ± 94 (PVN) and 1259 ± 126 (SON) for the paraffin tissue. Variations introduced by the method itself yielded a coefficient of variation of only 0.19. Furthermore, a non-significant negative trend with postmortem delay was found in cryostat tissue, but not in paraffin sections. No effect of fixation time was observed in the paraffin tissue. Both ways of tissue treatment have specific advantages and disadvantages that may be different for other probes or other brain areas. For ISH of a highly abundant mRNA like AVP in a very heterogeneous brain area such as the human hypothalamus, formalin-fixed paraffin-embedded tissue sections can be used for quantitative analysis of entire brain nuclei because of the small variation in this tissue, the remarkably good signal recovery (some 75% as compared to cryostat sections) and its practical advantages with regards to anatomical orientation, storage and sampling of the tissue.

Keywords: Hypothalamus; In situ hybridization; Vasopressin; Formalin fixation; Paraffin embedding; Cryostat section; Quantitative method

1. Introduction

Originally described by John et al. (1969) and Pardue and Gall (1969), in situ hybridization (ISH) has revolutionized the spatiotemporal analysis of gene expression and has become a widely employed method in neurobiology. Both in rat and human studies, ISH is applied generally on cryostat sections. When applying quantitative ISH on an extremely heterogeneous brain area such as the human hypothalamus (Swaab et al., 1993), the use of paraffin-embedded tissue would be of

great practical advantage, since serial sectioning is more difficult for cryostat sections, as is the storage of large numbers of such sections. Moreover, formalin-fixed paraffin-embedded human tissue of rare cases is usually more readily available, and in quantities that allow comparison between larger numbers of patients. Another advantage would be that in principle, it might become possible to use tissue that has been stored for a long time.

The present study investigated, therefore, the suitability of formalin-fixed paraffin-embedded human hypothalamic tissue for quantitative ISH in a comparative approach. Maximal ISH signals for vasopressin-mRNA (AVP-mRNA) in tissue sections of symmetric halves of 5 human hypothalami that were either quickly frozen

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or formalin-fixed paraffin-embedded, were compared by means of densitometry of film autoradiographs. AVP was chosen since it is abundantly present in 2 major nuclei of the hypothalamus, the supraoptic (SON) and paraventricular nucleus (PVN) (Swaab et al., 1993). AVP acts as an antidiuretic hormone on the kidney (Cunningham and Sawchenko, 1991), is involved in the stress response and has central actions (Rivier and Vale, 1983; Sofroniew, 1983). Densitometric analysis of systematically obtained sections through the SON and PVN was performed in order to estimate total amounts of AVP-mRNA present in the SON and PVN in each tissue half.

2. Materials and methods

2.1. Tissue preparation

Brains were obtained at autopsy from 5 female subjects ranging in age from 61 to 85 years. Four subjects had no neurological or psychiatric disease and one was an Alzheimer patient (Table 1). The hypothalamic area containing the SON and PVN was removed and cut in the sagittal plane through the 3rd ventricle yielding 2 symmetrical halves. One half was fixed for 10–35 days in 4% buffered formaldehyde at room temperature, whereas the other half was put in a small steel box to preserve tissue morphology, frozen immediately in liquid nitrogen and stored at -80°C for different periods of time with a maximum of 1 year before cutting. The formalin-fixed half was embedded in paraffin after which serial $10\text{ }\mu\text{m}$ frontal sections were cut on a Leitz microtome. Sections were mounted on amino-alkyl-silane (AAS) coated slides using a 0.5% bovine albumin solution (96–99%, Sigma) (Henderson, 1989) that was sterilized by filtration. The frozen hypothalamic half was serially sectioned on a cryostat at -15°C (Frigocut, Reichert-Jung) and $10\text{ }\mu\text{m}$ sections were stored at -20°C for about 22 months before hybridization. In order to determine the number of sections needed to estimate the total amount of mRNA in a nucleus accurately, an ad random starting point was chosen and 1 out of every 50 sections through the SON and PVN was taken from there for hybridization

on the basis of their location in the thionine series (Uylings et al., 1986; Gundersen and Jensen, 1987). It was attempted to keep the working conditions as RNase-free as possible by using sterile glasswork, autoclaved buffers and wearing gloves throughout the entire procedure (McCabe and Pfaff, 1989; Moorman et al., 1993).

2.2. Probe labeling and purification

Vasopressin probe (kindly donated by Dr. G. Mengod and Dr. J.M. Palacios (Sandoz Research Laboratories, Basel, Switzerland) was synthesized on a 380 Applied Biosystems DNA synthesizer, purified on 20% polyacrylamide/8 M urea preparative sequencing gel and stored at -20°C . The probe (hvp3), which did not contain homologies for oxytocin, consisted of an oligomer of 48 nucleotides complementary to bases 411–458 of the preprovasopressin precursor (Mengod et al., 1992). Controls for the specificity of the hybridization signal have been described previously (Mengod et al., 1990, 1992). Labeling mix was prepared according to the following protocol: 4 pmol of probe in $2\text{ }\mu\text{l}$ was added to $31\text{ }\mu\text{l}$ of distilled water (AD) and heated to 65°C for 5 min to uncoil the probe and then placed on ice. 40 pmol of ^{35}S - α -dATP (Spec. Act. $>1000\text{ Ci/mmol}$; $10\text{ }\mu\text{Ci}/\mu\text{l}$; Amersham) was thawed to room temperature and mixed with $3\text{ }\mu\text{l}$ 25 mM CoCl_2 and $10\text{ }\mu\text{l}$ of tailing buffer (Boehringer) containing 1 mM dithiothreitol (DTT), 500 mM sodium cacodylate (pH 7.2) and 10 mM CoCl_2 . The mix is incubated at 37°C for 5 min. Subsequently, $2\text{ }\mu\text{l}$ terminal deoxynucleotidyltransferase (TdT; $25\text{ U}/\mu\text{l}$; Boehringer, Mannheim) (Bollum, 1974) was added to the labeling mix and incubated at 37°C for 1 h. The tailing reaction was ended by addition of $400\text{ }\mu\text{l}$ Reagent A (100 mM Tris-HCL/1 mM ethylenediaminetetraacetic acid (EDTA)/1.4% triethylamine (v/v) that was sterilized by filtration (final pH: 7.7)). Nensorb columns (NENSORB-20, New England Nuclear) were used to purify the probe. The eluted probe was dried in a rotary evaporator (Savant) and stored at -20°C for 16 h until dilution. Determination of labeling efficiency yielded an average of 3–4 molecules of ATP coupled per molecule probe.

Table 1
Brain material used

Patient (no.)	Sex	Age (years)	PMD (h)	FIX	Brain wt (d)	Clinicopathological diagnosis/cause of death
1 91379	f	85	19	10	1067	Pulmonary embolus with secondary cardiac failure
2 91459	f	75	5h30	20	920	Pulmonary embolus
3 91380	f	65	9h30	10	1500	Mamma tumor, antiestrogen therapy, cardiac failure
4 91443	f	63	3h30	13	nd	Sudden cardiac death; Morbus Crohn
5 91271	f	61	4h15	35	846	AD, epilepsy, dehydration after swallowing disturbances

Abbreviations: f, female; PMD, postmortem delay (in hours); FIX, fixation time (in days); AD, Alzheimer's Disease; nd, not determined.

Both, the formalin-fixed paraffin-embedded and the non-fixed cryostat tissue sections were processed in the same hybridization run. Tissue sections were pre-treated before hybridization in order to enhance tissue permeability, decrease non-specific signal and reach an optimal signal intensity in both types of tissue. After deparaffination in xylene and rehydration through graded ethanols, the paraffin sections were dried briefly with a hairdryer and then, together with the rapidly thawed cryostat sections, they were post-fixed for 20 min in freshly prepared 4% paraformaldehyde in phosphate buffered saline (1 × PBS) (pH 7.5) (0.136 M NaCl; 1.47 mM KH_2PO_4 ; 8 mM Na_2HPO_4 ; 2.68 mM KCl per litre AD). Subsequently, sections were washed in dilutions of sterile 10 × concentrated PBS (10 × PBS) as described (Mengod et al., 1992), placed in 0.2 N HCl for 20 min and incubated for 15 min in proteinase K (PK; Sigma) at 37°C. Different PK concentrations with or without a preceding wash in 0.2 N HCl were tested on both tissues in order to obtain an optimal signal quality; i.e., a high signal-to-noise ratio in combination with good tissue morphology. Optimal concentrations were determined in pilot experiments and the following ones were used: 50 µg PK/ml PK buffer (10 mM Tris-HCl, 2 mM CaCl_2) for formalin-fixed paraffin-embedded tissue sections and 2 µg/ml for the cryostat sections. After PK treatment, all sections were washed in glycine buffer (2 mg/ml in 1 × PBS) to stop proteolysis, dehydrated through graded alcohols and delipidated for 5 min in chloroform and 2 min in ethanol 100% after which they were air-dried in a dust-free place.

2.3. Hybridization conditions

The labeled probe was diluted in a hybridization buffer containing 50% de-ionized formamide (Merck), 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumine (fraction V, RIA-grade), 500 µg/ml yeast tRNA, 250 µg/ml herring sperm DNA, 10% dextran sulfate and 30 mM DTT. Before it was added to the hybridization buffer, the herring sperm was denatured in boiling water for 2 min and then placed immediately on melting ice. The vacuum dried probe was reconstituted to a final concentration of about 2500 cpm/µl and 65 µl of hybridization buffer was added to each slide. In order to prevent evaporation, sections were covered with a piece of Nescofilm (Nippon Shohi Kaisha, Osaka, Japan) that was gently smoothed down to avoid the trapping of air bubbles. Hybridization took place overnight in a humidified box at 42°C.

Nescofilms were carefully removed by flotation in washing buffer containing 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Sections were washed

4 times, for 45 min each time, in washing buffer at 50°C and then dehydrated in 70% ethanol containing 0.3 M ammonium acetate (pH 7.5) and in 95% ethanol containing 0.3 M ammonium acetate (pH 8.2), both for 5 min and then air-dried.

For autoradiographic detection, β -max Hyperfilm (Amersham International, Buckinghamshire, UK) was firmly pressed on the slides for 145 h of exposure at room temperature. The film was then developed in Kodak D-19 developer, washed, fixed in sodium thio-sulfate (960 mM/l), washed again and air-dried.

2.4. Preparation of radioactive standards

In order to determine a relationship between the grey values in the autoradiograms read by the IBAS image analysis system and the amount of radioactivity, radioactive standards were prepared. By repeated dilution of the reconstituted labeled probe, 15 values were obtained, ranging from 20,000 cpm/µl to 160 cpm/µl of which a sample was counted in a scintillation counter. In addition, 3 times 1 µl of every value was spotted on 3 pieces of Whatman Filter paper 41 attached to a glass slide. These filters were air-dried overnight and placed amidst the hybridized sections in the cassettes.

2.5. Quantitative analysis

The in situ hybridization signal on the films was quantified using an IBAS image analysis system (Kontron, Germany) that was connected to a CCD camera (Sony 90C; XC 77 CE). In order to correct for differences in light temperature of the lamp and differences in spectral sensitivity of the CCD camera, monochromasy of the light was effected by placing a 560 nm small-band filter in the light path. A grey value transformation table was constructed by correlating the physical values of the radioactive standards, as determined in the scintillation counter, with the measured grey value of the spots on the film. Shading correction to compensate for non-uniform illumination in the microscope and differences in sensitivity of the camera was performed. A 100% transmission value was set in a non-irradiated field in a standardized part of every film.

In order to reach an adequate resolution with regard to the pixel density per unit area, films of all patients were measured comparing magnification factors of 25 × and 40 ×. 512 × 512 pixel large images with 256 grey levels were loaded into the IBAS and displayed on the image monitor. Subsequently, a thresholding procedure was started on the IBAS, producing a mask that coincided with the signal area on film (see Fig. 1A,B). The purpose of the mask was to select in an automated and reproducible way the exact area of blackening on the film not influenced by differences in background. Parameters for construction of

the mask were set on the basis of the size of a particle and local contrast on the film. In that way, differences in absolute signal intensity hardly influenced the final area of the mask. The use of a mask was chosen because in human nuclei such as the PVN and SON, cells are often dispersed over a relatively large area. Earlier pilot experiments (Dr. C.W. Pool, personal communication, data not shown) demonstrated that densitometry in an outlined area of interest without the use of a mask was very strongly influenced by the background within the outlined area.

Every mask that was constructed for an individual section was subsequently superimposed over the image allowing visual inspection. The area on film encompassing the structure of interest was roughly outlined by hand (Fig. 1B). Then, of the area on film covered by the mask, structure weighted mean density and total area of the mask inside the outline were measured.

For background correction, an inverted image of every loaded image was used consisting of the mean density of the area within the manually drawn outline from which the area of the somewhat dilated mask inside it was subtracted. Using this value, background values were calculated, yielding in total 3 final values per section; total area, structure weighted mean density and a background corrected, structure weighted mean density. Data were stored per section, per patient and per group.

For each tissue half, about 11 sections (cryostat sections: 11.6 ± 1.1 ; paraffin sections: 10.4 ± 0.4 (mean

$\pm$ SEM)) were used for hybridization. Films were analyzed in order to construct a profile of integrated density values per section through the entire PVN or SON. These values were calculated by multiplying the area of the mask and the background corrected, structure weighted mean density of the mask for every section. On the basis of the systematically obtained sections a profile through every nucleus was obtained. Determination of the total area under the curve through these profiles was subsequently used to estimate total amounts of radioactive label present in an entire nucleus; Total Integrated Weighted Density (IntWD). Likewise, the total area under the curve through the profile of area values yielded a measure for the volume of the nucleus; Total Integrated Area (IA) (see Table 2A).

Since the sampling frequency should not influence the shape of the curve and thus the estimate of integrated density and area, a 1-of-50 sampling was compared with a 1-of-100 sampling in order to see whether this would have changed the total area under the curve or the mean values and standard errors of every group. In addition, the coefficient of error (CE) was calculated in order to provide information on the precision of the sampling procedure (Gundersen and Jensen, 1987; McCabe and Bolender, 1993).

Furthermore, the variation introduced by the ISH procedure itself was determined. In order to do so, we chose the PVN of the paraffin-embedded tissue half of patient no. 91380 since it displayed a very regular,

Table 2

Patient (no.)	PMD (h)	FIX (days)	CRYPVN			PARPVN			CRYSON			PARSON		
			IntWD	CE	IntA	IntWD	CE	IntA	IntWD	CE	IntA	IntWD	CE	IntA
A: 1-of-50 sampling ^a														
91379	19	10	650.91	0.07	1.24	1041.85	0.06	1.74	1700.60	0.08	2.48	1403.32	0.09	2.14
91380	9.5	10	689.01	0.04	1.47	835.12	0.04	1.68	1698.75	0.04	2.67	1518.68	0.05	2.76
91443	3.5	13	1653.48	0.08	2.56	671.30	0.10	1.93	3100.92	0.03	3.81	1094.34	0.07	2.13
91459	5.5	20	1244.31	0.04	2.11	665.45	0.09	1.56	2929.70	0.07	4.51	845.61	0.06	1.96
91271	4.2	35	2249.01	0.16	3.06	1128.16	0.08	2.74	3267.56	0.06	3.15	1438.63	0.09	2.81
Mean	4.5	17.6	1297.35	0.07	2.09	868.47	0.07	1.93	2539.18	0.05	3.32	1259.66	0.07	2.36
± SEM	1.5	4.7	302.20	0.02	0.30	94.3	0.01	0.2	346.60	0.008	0.4	126.1	0.007	0.2
B: 1-of-100 sampling ^b														
91379	19	10	616.35	0.2	1.20	1033.30	0.09	1.84	1421.85	0.12	2.21	1233.20	0.1	2.02
91380	9.5	10	690.52	0.09	1.63	755.04	0.05	1.60	1670.35	0.15	2.54	1423.04	0.1	2.69
91443	3.5	13	1498.47	0.11	2.50	582.42	0.11	1.62	3162.61	0.04	3.87	1043.85	0.1	2.08
91459	5.5	20	1235.02	0.08	2.14	609.42	0.07	1.40	2367.56	0.06	3.92	769.30	0.12	1.90
91271	4.2	35	2733.43	0.34	3.47	929.58	0.18	2.29	3118.92	0.10	2.70	1854.89	0.14	2.99
Mean	4.5	17.6	1354.20	0.16	2.19	781.60	0.10	1.75	2347.62	0.09	3.05	1264.44	0.11	2.34
± SEM	1.5	4.7	382.2	0.04	0.40	88.1	0.02	0.20	358.60	0.019	0.40	182.7	0.007	0.2

^a Table displaying per patient: IntWD, integrated weighted density, and IntA, integrated area values, representing total amounts of radioactivity and total volume of the signal measured, respectively, for a complete SON or PVN in the cryostat (CRY) and paraffin (PAR) tissue on the basis of a 1-of-50 sampling. Furthermore depicted are the coefficient of error (CE) values per patient and per condition (Gundersen and Jensen, 1987; McCabe and Bolender, 1993; see text for details). *Abbreviations:* PMD, postmortem delay; FIX, fixation time; PARPVN, paraventricular nucleus (PVN); formalin-fixed paraffin-embedded; CRYPVN, PVN in the cryostat sections; PARSON and CRYSON, same for the supraoptic nucleus (SON).

^b Same as (A), but for a 1-of-100 sampling.

almost horizontal profile (Fig. 2B). Ten consecutive sections were mounted, hybridized according to the same protocol and analyzed.

3. Results

Using the current protocol, a strong hybridization signal was observed in both the non-fixed cryostat as well as the formalin-fixed paraffin material of all 5 patients, localized exclusively in the SON and PVN (Fig. 1). With the naked eye, no obvious differences could be observed in the signal on film obtained from

cryostat or paraffin material. Patients data are depicted in Table 1.

When comparing the $25\times$ magnification factor with the $40\times$ for analysis, no differences in total amounts of signal were found. Since in the $25\times$ magnification, 1 pixel represented $13.6\text{ }\mu\text{m}$, whereas in the $40\times$ magnification this was $8.1\text{ }\mu\text{m}$, the $40\times$ was chosen. This magnification allowed higher resolution on the IBAS image and the inclusion of a complete nucleus per image in almost all cases. With regard to the PK pretreatments, the concentrations used turned out to give maximal signal quality without tissue damage; higher concentrations (e.g., $100\text{ }\mu\text{g/ml}$ in the paraffin tissue or $20\text{ }\mu\text{g/ml}$ in the cryostat tissue) did not improve signal quality any more and induced tissue damage. Lower concentrations (e.g., $2\text{ }\mu\text{g/ml}$ in the paraffin tissue) yielded a weak, if any, signal compared to other concentrations (data not shown).

The results of quantification of the autoradiograms are shown in Fig. 2A–D and represent total amounts of radioactivity in profiles of 2 representative patients through the SON and PVN. Furthermore, in Table 2A,B, IntWD and IA values are shown, representing measures for the total amounts of radioactivity in an entire nucleus and for the total volume of the hybridization signal, respectively, in a 1-of-50 (Fig. 2A) and a 1-of-100 (Fig. 2B) sampling. Also, for every patient, coefficient of error (CE) values are depicted that were calculated on the basis of the product of the background corrected, structure weighted mean density values and signal area per section per patient (see also quantitative analysis section in Section 2).

As to the sampling strategy chosen (i.e., 1 of every 50 sections; data represented in Table 2A), a selective sampling of 1 of every 100 sections from the same data set yielded highly comparable IntWD values with regard to the mean values and standard errors of the groups (see Table 2A,B; e.g., CRYPVN (1-of-100): 1354.2 ± 382.2 (mean $\pm$ SEM vs. CRYPVN (1-of-50): 1297.35 ± 302.2), indicating that the sample strategy used in the present analysis (i.e., 1-of-50) had not influenced the IWD and IA values strongly and was thus sufficient. CE values, in general, were low in the 1-of-50 sampling (mean values of the groups ranging from 0.05 to 0.07; Table 2A), whereas somewhat higher values were obtained in the 1-of-100 sampling (mean values ranging from 0.09 to 0.16; Table 2B).

Considerable differences between patients were observed in total amounts of radioactivity as well as differences in the shape of the profiles. The shape of the PVN profile of patient no. 91380, e.g., most probably due to a different orientation of the cutting plane, displayed an almost horizontal curve (Fig. 2B), whereas the PVN profile of patient no. 91443 (Fig. 2D) yielded a clearly different profile in the paraffin tissue. Moreover, for an entire nucleus, IntWD values showed

Fig 1. A: photomicrograph depicting ^{35}S -AVP-mRNA hybridization signal on film in the human SON and PVN (paraffin section of patient 90379). Bar: 1 mm. B: photomicrograph depicting the same nuclei as in (A), including the constructed mask and the manually made outline.

differences per patient of even a factor 3 in the cryostat tissue (e.g., Table 2A, CRYPVN; 1297.35 ± 302.2 (mean $\pm$ SEM)); considerable less variation in IntWD values between patients was present in the paraffin tissue (e.g., Table 2A, PARSON; 1259.66 ± 126.1 (mean $\pm$ SEM)).

Optimizing signal quality in both tissues resulted in total amounts of signal that in general, were lower in the paraffin material as compared to the cryostat tissue (Figs. 2–3; Table 2A). The range of this loss of signal in formalin-fixed paraffin-embedded tissue as compared to cryostat sections varied from 10% to 68% for the SON and from 17% to 49% for the PVN (Table 2A). In Table 3, ratios of IntWD values in paraffin over those in cryostat sections are depicted. As a mean, some 15% of the signal for the PVN (ratio paraffin/cryostat: 0.846 ± 0.23 (mean $\pm$ SEM)) and some 40% for the SON (ratio: 0.563 ± 0.12) was lost (Table 3). Comparison of these data using the Student's *t* test for paired data did not yield any statistically significant difference between the SON and PVN ratios ($P = 0.1$).

mRNA distribution in the paraffin SON and PVN tended to be similar to that in cryostat material. The

Table 3 Ratios

Patient				
91379	PVN	1.60	SON	0.83
91380	PVN	1.20	SON	0.89
91443	PVN	0.40	SON	0.35
91459	PVN	0.53	SON	0.31
91271	PVN	0.50	SON	0.44
Mean ($\pm$ SEM)	PVN	0.846 ± 0.23	SON	0.563 ± 0.12

Ratios of IntWD values in paraffin over those in cryostat tissue.

Abbreviations: PVN, paraventricular nucleus; SON, supraoptic nucleus.

shape of the curve in the cryostat profile is generally reflected in the paraffin half (e.g., Fig. 2A: SON and Fig. 2C,D: SON and PVN) although smaller. Fluctuations between consecutive sections were more obvious in the cryostat tissue (Table 2A). The variation introduced by the ISH procedure itself, as measured in 10 consecutive sections in the paraffin PVN of patient no. 91380, yielded a coefficient of variation of only 0.19 (349.5 ± 21 (mean $\pm$ SEM)). When plotting IntWD values versus the postmortem delay (PMD), no relation-

Fig. 2. Graphs depicting profiles of total amounts of radioactivity (RA) in AU per section for paraffin (par) and cryostat (cryo) through the SON and PVN of 2 representative patients: no. 91380, SON (A), PVN (B); and no. 91443, SON (C) and PVN (D). Values per section are based on the product of signal area and background corrected, structure weighted mean density values (see Section 2 for further details).

Fig. 3. Graphs depicting: (A) integrated weighted density (IntWD) values in AU, representing total amounts of radioactivity for a complete SON or PVN in the cryostat (CRYSON and CRYPVN) and (B) in the paraffin (PARSON and PARPVN) tissue per patient (see Table 1 for details) plotted vs. the PMD. A non-significant negative trend is present in the cryostat tissue ($P = 0.07$ (SON) and $P = 0.13$ (PVN)). No clear effect of the PMD is observed in the paraffin tissue ($P = 0.48$ (PVN) and $P = 0.47$ (SON)).

ship ($P = 0.48$ (PVN) and $P = 0.47$ (SON)) was found in the formalin-fixed paraffin-embedded tissue (Fig. 3B), whereas in the cryostat tissue a non-significant trend ($P = 0.07$ (SON) and 0.13 (PVN)) appeared to be present between these 2 parameters (Fig. 3A). Material from the 3 patients with a short PMD (< 6 h), in particular the PVN in the cryostat material, had the tendency to display higher IntWD values than material from patients with longer PMDs. Using linear and non-linear regression analysis, however, no statistically significant correlation was found. In addition, no correlation was observed between IntWD values and fixation time for the formalin-fixed paraffin-embedded tissue ($P = 0.46$ (PVN) and $P = 0.98$ (SON) (data not shown)).

4. Discussion

The measurement of mRNA levels in hypothalamic nuclei provides a tool for the assessment of the level of gene expression in human autopsy material. The present study confirms the applicability of qualitative and quantitative ISH for, for example, AVP-mRNA on frozen tissue (Meister et al., 1990; Mengod et al., 1991; McCabe et al., 1993; Murayama et al., 1993; Reppert and Uhl, 1988; Sukhov et al., 1993). In addition, the present paper shows that systematically obtained sections from routinely formalin-fixed paraffin-embedded tissue can be used for quantification of relative amounts of mRNA as reflected by total amounts of radioactivity in an entire human hypothalamic nucleus.

Considerable variation was found between the patients (Table 2A). The variability of the method did not contribute to any relevant degree to this variation since the CV was found to be 0.19. In general, the cryostat material of these 5 patients displayed higher total amounts of bound radioactivity (IntWD) and higher variation than the paraffin tissue. In other studies, within-group differences in hybridization densities have been reported (Rivkees et al., 1989; Mengod et al., 1991; McCabe et al., 1993). Ante- and postmortem factors such as disease, agonal state, stress, hypoxia, dehydration state, PMD, type and duration of fixation and storage time of the tissue may contribute to the variation observed in RNA levels in human brain material (Burke et al., 1991; Barton et al., 1993). 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, the half-life of AVP-mRNA in postmortem rat brain was shown to be approximately 16 h (Noguchi et al., 1991). Also, Arai et al. (1989) noted a significant decrease in AVP-mRNA amounts in rat brains fixed 24 h after death. Relatively few ISH studies on postmortem effects on human brain material have been performed. Using ISH, several human mRNAs have been localized after PMDs of up to 40 h (Mengod et al., 1990; Solà et al., 1993). In addition, no significant correlation was found between the density of the hybridization signal and the PMD (range: 2.5–66 h) in a comparison of pro-opiomelanocortin (POMC)-mRNA levels in pituitaries between controls and several diseased patients (Mengod et al., 1991). It must be noted, however, that these results were based upon single density measurements instead of on the basis of integrated profiles of entire structures. The present study suggests that the total amount of signal in cryostat sections does decrease with increasing PMD (Fig. 3A). Due to the distribution and the small number of observations, no general extrapolations or statistical conclusions can be drawn from these data. It does suggest, however, that for quantitative analysis of AVP-mRNA in frozen human brain tissue, careful

matching for PMD should be performed. The relatively large total amounts of signal found in cryostat tissue of the 3 shortest postmortem times (i.e., 3.5, 4.25 and 5.5 h) was not found in the paraffin-embedded halves of the same patients. This indicates that loss of signal in this tissue is likely related to the fixation and paraffin-embedding procedures. After the first few hours, however, the loss seemed less prominent. Although only a few time points are recorded, no further decrease in signal was detected after 6 h PMD. This lack of a clear PMD effect in the paraffin tissue remains unexplained. Furthermore, when comparing paraffin with cryostat tissue, ratios of total amounts of radioactivity for paraffin tissue over cryostat tissue show that apparently more signal is lost in the SON than in the PVN (Table 3), which may be related to differences in the total amount of AVP-mRNA present between these nuclei. More extensive research on these matters may provide explanations for the present findings.

From immunocytochemical studies it was known that prolonged fixation in, e.g., formalin, may induce extensive cross-linking which can prevent antibodies from reaching their antigen (Ravid et al., 1992; Lucassen et al., 1993). The same may hold for oligonucleotides, and even before/after PK or HCl treatments, differences in tissue matrix may still contribute to differences in signal intensity. In the present study no clear correlation between total amount of signal and fixation time was found, even though 2 patients who showed comparable total amounts of signal between paraffin and cryostat sections (Table 2A, Table 3 and Fig. 2A; no. 91379; SON and no. 91380; PVN and SON), were both fixed for the shortest period of time (i.e., 10 days). This suggested that fixation time may be important for the recovery of the AVP signal in paraffin tissue. However, statistical analysis of data obtained using oxytocin (OXT) and corticotropin-releasing hormone (CRH) probes on comparable material did not yield a significant correlation between fixation time and total amount of signal (S.E.F. Guldenaar, personal communication; F.C. Raadsheer et al., 1994).

Storage of frozen tissue at -70°C for more than 5 years may compromise its use for molecular biological studies (Leonard, 1993). However, for ISH procedures, storage of frozen tissue for more than 6 years at -80°C was reported not to influence hybridization signal intensity significantly (Mengod et al., 1992). Storage at higher temperatures, e.g., -25°C , could, however, lead to relatively rapid mRNA degradation (S.E.F. Guldenaar, personal communication). From other studies at our Institute, it became clear, that a negative correlation seems to be present between storage time and total hybridization signal intensity in the paraffin-embedded tissue (S.E.F. Guldenaar, unpublished results; Raadsheer et al., 1994). However, since all the tissue in our study was obtained in the same

year and ISH was performed in the same session, storage time could not have interfered with our experiments. As to the effect of storage time on ISH signal in general, it has been reported that some mRNAs may be more sensitive to specific ante- and postmortem factors than others (Barton et al., 1993). For other probes, the effects of these factors on the ISH signal obtained are thus not necessarily equal to those of AVP. Raadsheer et al. (1994), e.g., did not find a correlation between the total amounts of CRH signal and PMD, nor with fixation time or the age of the patient. However, clear significant differences could still be found between the patient groups studied. Our results may, therefore, encourage pilot experiments to elucidate whether there is any correlation with, e.g., PMD or storage time, before ISH analysis on human autopsy tissue with a particular probe is started.

In the present study, paraffin sections were compared with cryostat sections of the same nuclei of the same patient. In the formalin-fixed paraffin-embedded tissue, about 27.5% of the total amounts of radioactivity appears to be lost or cannot be reached without loss of tissue integrity in comparison to the cryostat tissue (Table 3) which is in agreement with Wilcox (1993). Although some loss is present, the signal in paraffin tissue, in general, faithfully reflects the profiles of the cryostat sections (Fig. 2C,D). Furthermore, our data confirm earlier findings (Rivkees et al., 1989) that (de)hydrational state increases AVP-mRNA levels in frozen human brain tissue; patient no. 91271 suffered from dehydration (Table 1) and indeed displayed high IntWD values in the cryostat material (Table 2A). In addition, rat studies demonstrated that strongly elevated AVP-mRNA levels were present primarily after chronic osmotic stimulation; Zingg et al. (1986) therefore suggested that their rise should be expected in terms of days rather than hours following dehydration.

In conclusion, for quantitative analysis of ISH signal in entire brain nuclei, both tissue treatments have their specific advantages and disadvantages. Cryostat tissue appeared to yield higher signal densities than paraffin, but with larger variations. Also, the strong effect of PMD on total amounts of radioactivity observed in this tissue calls for careful matching which may be a problem of a more practical nature, but highly relevant for this type of study. Formalin-fixed paraffin-embedded tissue, on the other hand, has certain practical advantages as far as systematic sampling, anatomical orientation and storage of, e.g., large amounts of sections is concerned. Although signal loss was found due to the fixation and paraffin-embedding procedures, quantification of the total amount of radioactive signal obtained in formalin-fixed paraffin-embedded tissue in general, faithfully reflects the profile obtained in cryostat tissue with a recovery of about 73% for this particular probe as compared to cryostat sections. For other

probes, however, these features may have to be re-investigated.

Acknowledgements

We are indebted to Dr. A. Probst from the Institute of Pathology, Basel, Switzerland and the Netherlands Brain Bank (Coordinator: Dr. R. Ravid) for providing the human material. We further thank mr. J.J. van Heerikhuize for technical suggestions and valuable comments to the manuscript and Dr. M.A. Hofman and Dr. R.W.H. Verwer for their methodological and statistical advices. Also, we are grateful to Dr. A.F.M. Moorman of the Department of Anatomy and Embryology, Academic Medical Centre, Amsterdam, for helpful and fruitful discussions. Furthermore, we thank mr. G. van der Meulen for photography and miss W. Verwey for corrections of the English. E.G. was supported by the Sandoz Foundation for Gerontological Research (Grant m.SF 194), by the Brain Foundation of the Netherlands and the Innovation Fund of the Royal Dutch Academy of Sciences. P.J.L. was supported by the Netherlands Organization for Scientific Research (NWO, dpt. Medical Sciences: grant no. 900-552-083). F.C.R. by the Graduate School Neurosciences Amsterdam and S.E.F.G. by the Innovation fund of the Royal Dutch Academy of Sciences.

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