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A quantitative in situ hybridization protocol for formalin-fixed paraffin-embedded archival post-mortem human brain tissue

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ABSTRACT

The use of radioactive in situ hybridization (ISH) to *quantitatively* determine low-to-moderate abundant mRNA expression in formalin-fixed, paraffin-embedded archival post-mortem human brain tissue is often limited by non-specific-deposits, visible as speckles. In the present study, optimal hybridization conditions were achieved for quantifying the mRNA expression of histidine decarboxylase (HDC) by a number of alterations in a routine protocol, which included (1) during purification of the oligo-probes, glycogen was omitted as a carrier for precipitation, (2) after precipitation, the labeled probe contained within the pellet was first dissolved in water instead of in hybridization buffer (HBF), (3) during hybridization, the dithiothreitol (DTT) concentration was increased from 200 to 800 mM in HBF, and (4) stringencies during hybridization and post-hybridization washes were increased by increasing the temperature. The effect of the adjustment was quantified on adjacent sections from 18 subjects (9 with Parkinson's disease and 9 controls), by comparing the data from the standard and new protocol. The results showed that the improved protocol brought about significantly clearer background with higher signal-to-noise ratios ($p = 0.001$). We propose that this protocol is also applicable for detection of other lower-abundant genes in human brain tissue and probably in other tissues as well. In the present study, this is not only illustrated for HDC ISH, but also for corticotrophin-releasing hormone mRNA expression in the hypothalamic paraventricular nucleus.

1. Introduction

In situ hybridization (ISH) allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections. This technique is widely used to detect the expression of specific genes at the mRNA level. When relative quantitative comparison between experimental groups is needed, radioactive ISH still holds the unique advantage of signal quantification. Although frozen tissue sections are often used for ISH, paraffin-embedded tissue sections have great practical advantages for anatomically complex structures such as the hypothalamus with regard to anatomical orientation, tissue storage and the study of archival material. In the last decade, our group has successfully developed radioactive ISH on formalin-fixed, paraffin-embedded human brain tissue sections to detect mRNA expression of e.g. vasopressin, oxytocin, tyrosine hydroxylase, corticotropin-releasing hormone (CRH), neuropeptide Y, agouti-related protein, and thyrotropin-releasing hormone [1–9]. However, when the standard ISH protocol was used for the detection of a gene of low-to-moderate abundance, background issues usually arise in the form of non-specific deposits of radioactivity, seen as speckles on autoradiographic film.

There are two sources of non-specific labeling of tissue following ISH: (1) cross-hybridization to related sequences other than the target, and (2) probe binding to non-RNA components in the tissue. While cross reaction can be limited or eliminated by a combination of careful probe design and manipulation of hybridization and washing conditions [10], interactions with non-RNA components are governed by chemical interactions which cannot be effectively addressed at times only by alteration of stringency. Therefore, optimizing ISH protocols represents a balance between conditions required for selective hybridization and conditions that limit probe binding to non-RNA components.

To address the background problems consisting of non-specific deposits, we introduced a number of alterations in the 'routine' protocol, illustrated here by the optimization procedure for

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quantitative ISH study of histidine decarboxylase (HDC) mRNA expression, the rate limiting enzyme for histamine production, in the human hypothalamic tuberomamillary nucleus (TMN), using paraffin-embedded brain tissue sections. The TMN in the posterior hypothalamus is the exclusive location of histaminergic neurons that send their fibers to almost all the regions of the human brain [11–14]. As a neurotransmitter in the central nervous system, histamine holds a key position in the regulation of basic body functions, including attention, the sleep–wake cycle, energy and endocrine homeostasis, synaptic plasticity and learning [15]. In addition, the TMN may also play a role in brain diseases such as epilepsy, Alzheimer's disease (AD), Parkinson's disease (PD) [15]. Changes in the TMN are presumed to occur in PD on the basis of the extensive accumulation of the characteristic neuropathological PD lesions, i.e. the Lewy bodies and Lewy neuritis in this area [16,17]. We aimed to optimize the protocol for reliably detecting changes in HDC-mRNA expression in the TMN of PD patients, using formalin-fixed, paraffin-embedded archival post-mortem human brain tissue. To this end, we introduced a few alterations into the routine protocol used in our group. We believe that this improved protocol has a general applicability for other low-to-moderate abundant genes in formalin-fixed, paraffin-embedded archival post-mortem human brain tissue, as shown here for the expression of CRH-mRNA in the hypothalamic paraventricular nucleus (PVN).

2. Materials and methods

A quantitative ISH study protocol can be divided into 5 consecutive stages: tissue preparation, probe labeling and purification, hybridization, post-hybridization washes, and autoradiographic detection. Variables involved in these different stages that might affect the results were tested alone or in combination. Based upon these tests, an improved ISH protocol was established and validated for film-quantification of HDC-mRNA expression in the TMN of PD patients and controls.

2.1. Human brain material

Brain material was obtained from the Netherlands Brain Bank (Head Dr. I. Huitinga) following permission from the patient or the next of kin for brain autopsy and for the use of the brain material and clinical data for research purposes. Eighteen brain samples were studied, 9 of whom were PD patients with an age range of 56–87 years and 9 were control subjects matched for sex, age, season and clock time of death, brain weight, post-mortem delay, fixation time, and storage time in paraffin (Table 1). The hypothalami were dissected and fixed in 0.1 M phosphate buffered 4% w/v formaldehyde (pH 7.2) for 1–2 months. Following fixation, tissue was dehydrated in graded ethanol series, embedded in paraffin and serially cut in frontal sections (6 μm) on a Leitz microtome and stored at room temperature. Every 100th section was stained with thionine (0.1% w/v thionine in acetate buffer, pH 4) throughout each hypothalamus in order to localize the TMN. The rostral–caudal length of the TMN was defined as the distance between the most rostral and most caudal section which contained three or more typical TMN neurons. A semi-quantitative study of the distribution of TMN cells showed that the highest number of TMN cells was generally found in the middle, i.e. at 50–60% level of the rostral–caudal length of the TMN (data not shown). Consecutive sections from this mid-portion of the TMN of two control subjects (Subject #00-022 and subject #97-100) were used for a pilot experiment that investigated the effect of the modifications. To validate the observed effects, two consecutive sections from the mid-portion of the TMN of each of the 18 subjects were mounted. One of these sections was processed according to the routine protocol while the adjacent section was subjected to the modified procedure.

2.2. In situ hybridization for HDC-mRNA

2.2.1. Probe preparation

A 45-mer oligonucleotide probe (antisense probe: 5′ GGC AGG ACT CAT CAG CAT CGG GCT CAG ACG TTT TCA TTT CCA GGA 3′) was targeted to bases 647-603 of the human HDC-mRNA sequence [18], adapted from the probe described in [19]. It should be noted that according to our experience, probes of 40–55 nucleotides (nt) give much better results for ISH than probes of about 15–25 nt which are typically too small for optimal ISH. The shorter probes easily cause a non-specific signal. On the other hand, probes larger than 55 nt usually cause failure of ISH, probably because of the difficulty to penetrate the tissue (personal observations). The probe

Table 1
Clinico-pathological data of subjects.

	NBB	Age	Sex	MON	CTD	PMD	BW	FD	ST
PD									
	00-034	86	M	3	06:30	510	1178	45	7
	00-102	56	M	8	19:05	305	1515	37	7
	00-115	70	M	10	02:10	545	1258	35	7
	02-013	80	F	2	05:30	<1410	1254	30	5
	02-057	62	M	6	08:00	555	1700	43	5
	02-064	87	M	7	16:41	440	1166	36	5
	93-064	73	F	10	N.A.	2460	1165	34	15
	95-005	78	F	1	12:55	250	1110	31	12
	98-043	81	F	3	10:30	250	1330	43	9
Mean ± SD		74.8 ± 10.6				747.2 ± 732.4	1297.3 ± 193.1	37.1 ± 5.4	8 ± 3.5
Control									
	00-022	83	F	2	21:00	465	1102	34	7
	00-142	82	F	12	15:10	330	1280	36	7
	95-093	78	M	9	4:00	420	1440	29	12
	95-106	74	M	11	13:00	480	1317	60	12
	97-100	76	M	4	22:00	<1140	1315	133	10
	97-156	77	F	11	8:30	160	1235	47	10
	98-016	82	F	2	09:45	645	1078	35	9
	98-127	56	M	8	15:45	325	1522	35	9
	99-101	69	M	8	3:30	1155	1352	41	8
Mean ± SD		75.2 ± 8.8				598.9 ± 414.3	1293.4 ± 143.5	50.0 ± 32.4	9.3 ± 1.9

Note. BW, brain weight; CTD, clock time at death; F, female; FD, fixation time in days; M, male; MON, month of death; N.A., not available; NBB, Netherlands brain bank number; PMD, post-mortem delay in minutes; SD, standard deviation; ST; storage time in years.

was checked for cross homologies with other known sequences using BLAST [20], but none were found in the databases at the National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), USA. In addition, a HDC sense oligonucleotide probe directed against the same region of the HDC-mRNA sequence (5′ TCC TGG AAA TGA AAA CGT CTG AGC CCG ATG CTG ATG AGT CCT GCC 3′) served as a negative control.

2.2.2. Routine protocol

2.2.2.1. Tissue preparation. Sections were mounted on RNase-free 2% amino-alkyl-silane (AAS) coated slides and dried in a stove set at 37 °C for at least 2 days. Following deparaffinization in xylene, rehydration via a descending series of ethanol and brief rinses in phosphate buffered saline (PBS), sections were autoclaved for 20 min at 120 °C under a pressure of 1 bar in 0.01 M citrate buffer (pH 6.0). This method of antigen unmasking was shown to greatly enhance signal sensitivity for HDC-mRNA in previous pilot ISH experiments. After cooling down and delipidation in PBS containing 0.1% Triton X-100, sections were ready for hybridization.

2.2.2.2. Probe labeling and purification. The probe was 3′-end labeled using terminal deoxynucleotidyltransferase (Cat. No. 03333566-001, Roche Diagnostics, Bazel, Switzerland) and [α-^{35}S] dATP (Cat. No. NEG734H, PerkinElmer Life and Analytical Sciences, MA, USA) as described earlier [5]. After the tailing reaction (volume = 20 μl), the following reagents were added to purify labeled probe through ethanol precipitation: 21 μl sterile water, 1 μl glycogen solution (20 mg/ml, Cat. No. 901393, Roche Diagnostics, Bazel, Switzerland), 5 μl 3 M sodiumacetate (pH 5.5) and 2 μl 1 M $MgCl_2$. After mixing, 1 μl 50 mM dithiothreitol (DTT, Cat. No. D-9779, Sigma–Aldrich, St. Louis, USA) was added into the mix and a sample was taken to assess the total amount of radioactivity. Subsequently, 18.2 μl 7.5 M ammonium acetate and 136 μl cold 100% ethanol were added and precipitation occurred on dry ice. The following day, the mix was spun down for 30 min at 14,000 rpm to separate the labeled probe (pellet) from unincorporated ^{35}S-dATP label (supernatant). Finally, the pellet was dissolved in hybridization buffer (HBF: 0.5 M NaCl, 1 × Denhardt's solution, 10 mM Tris–HCl, 1 mM EDTA, 10% dextran sulphate, 0.5 mg/ml yeast tRNA, 50% formamide, 200 mM DTT).

2.2.2.3. Hybridization, post-hybridization washing and autoradiographic detection. Hybridization, post-hybridization washes and autoradiographic detection were performed according to the protocol described previously [4], except for the following differences: (1) 70 μl of HBF containing approximately 1×10^6 cpm of labeled antisense HDC probe was applied to each section and hybridized overnight at 37 °C; (2) The next day, coverslips were removed in 2 × standard saline citrate (SSC) at 37 °C and sections were washed sequentially for 30 min at 41 °C in 1 × SSC, 2 × 15 min at 41 °C in 0.1 × SSC and finally 2 × 15 min at room temperature (RT) in 0.1 × SSC; and (3) Films were developed after an exposure time of 2 weeks while sections were exposed to autoradiographic emulsion for 6 weeks in order to analyze the signal microscopically, at the single-cell level. It should be noted here that emulsion on film is optimized for macroscopy, while dipping emulsion is particularly optimized for microscopy. In our experience a 3–4 times longer exposure times is needed for the dipped emulsion than for the film emulsion to give satisfying results.

2.2.3. Improved protocol

Several variables were modified individually or in combination to improve the routine protocol mentioned above. These modifications aimed at suppressing the non-specific deposits by enhancing resuspension of the pellet that contained the labeled probe after ethanol precipitation. To achieve this, glycogen was omitted as a carrier of precipitation, while afterwards, the pellet was dissolved in water, prior to dilution in HBF, instead of resuspending it directly in the highly viscous HBF as prescribed by the routine protocol.

Further changes in the ISH procedure comprised increased stringencies during hybridization and post-hybridization washes. During hybridization, the temperature was raised from 37 to 42 °C, while DTT concentrations in HBF were increased from 200 to 800 mM. Post-hybridization washing temperatures were increased from 41 to 46 °C, whereas standard saline citrate (SSC) concentrations were decreased by half, i.e. 0.5 × SSC and 0.05 × SSC compared to 1 × SSC and 0.1 × SSC in modified and routine conditions, respectively.

The effects of the abovementioned modifications on signal-to-noise ratio were explored in a pilot study, employing consecutive sections from subjects #00-022 and #97-100. After having implemented the effective alterations into the routine procedure, 18 sections from nine PD and nine control subjects were subsequently used for a quantitative study of HDC-mRNA expression in the TMN for validation purposes and compared with adjacent sections, treated according to the routine protocol.

In addition, we had met similar problem of non-specific-deposits visible as speckles without specific signal in CRH-mRNA detection with the routine protocol (see Section 3, Fig. 4A), the improved protocol set up in the present study was thus used to detect the expression CRH-mRNA in the hypothalamic PVN, with a 48-bp oligonucleotide probe complementary to bp 1853–1900 of the human preproCRF gene (GenBank V00571) (antisense probe: 5′ AAT AAT CTC CAT GAG TTT CCT GTT GCT GTG AGC TTG CTG TGC TAA CTG 3′; sense probe: 5′ CAG TTA GCA CAG CAA GCT CAC AGC AAC AGG AAA CTC ATG GAG ATT ATT 3′)[21].

2.3. Quantitative analysis of HDC-mRNA in situ hybridization

Sections were apposed directly to autoradiographic film and exposed for 2 weeks. Thereafter, films were developed for 3–4 min in Kodak D-19 developer (Eastman Kodak Company, Rochester, NY, USA) and fixed in Kodak Maxfix for 5 min.

The HDC-mRNA signal was quantified with radioactive standards. The protocols of densitometry and quantification had been published before [5]. In short, the gray values of the film autoradiograms were analyzed by computer-assisted densitometry using Image Pro Plus (Media Cybernetics, Inc.) and corresponding software developed at our institute. The relationship between the gray values and the amount of radioactivity was assessed by using radioactive standards [1]. In addition, shading correction was performed to compensate for non-uniform illumination in the microscope and differences in sensitivity of the camera. A 100% transmission value was set in a non-irradiated field in a standardized part of every film. Subsequently, a thresholding procedure was started to produce a mask that coincided with the signal area on film, the purpose of which was to select in an automated and reproducable way the exact area of blackening on the film without influence of different backgrounds. Every mask that was constructed for an individual section was subsequently superimposed over the image allowing visual inspection. The TMN area on film was roughly outlined by hand. Then, 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 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: the total area of TMN, the structure-weighted mean density and a background corrected, structure-weighted mean density. The labeled area of the TMN and the structure-weighted,

background-corrected mean density of each section were used to estimate the amount of radioactive label in the TMN of each section ($Density_{TMN}$) that was a relative measure for the amount of HDC-mRNA. In the present study, the ratio of $Density_{TMN}$ to the density of its background ($Density_{TMN}/Density_{background}$) was used to assess the improvement of the protocol, in addition to the absence of non-specific speckles.

2.4. Statistical analysis

Differences in $Density_{TMN}/Density_{background}$ between the routine and improved protocol were analyzed by the Wilcoxon test. In addition, the association between parameters was examined with the Spearman correlation coefficients. A significance level of 5% (two-tailed) was used in all statistical tests.

3. Results

3.1. Specificity of HDC-mRNA in situ hybridization

Support for specificity of HDC-mRNA ISH signal came from the exclusive location of labeled cells in the TMN, observed on both film autoradiograms (Fig. 1A) and after emulsion autoradiography (Fig. 1B). Using emulsion autoradiography in combination with thionin-counterstaining, which enable signal analysis at the level of single cells, most of the characteristic, large TMN cells were masked with black silver grain deposits, showing medium to heavy labeling (Fig. 1B). In addition, specificity was supported by the failure of the labeled sense probe to show the signal in adjacent sections (Fig. 1C and D, respectively).

3.2. Improved protocol

Changes in the routine protocol which contribute significantly to a reduction of the non-specific speckles included dissolving the pellet in water prior to dilution in HBF to improve resuspension of a labeled probe (Fig. 2 B vs. A) and increasing the concentration of DTT in HBF from 200 to 800 mM (Fig. 2C vs. A). Raising the hybridization temperature from 37 to 42 °C, with the increase of post-hybridization washing temperature from 41 to 46 °C accordingly, also decreased the background, although this effect seemed relatively minor (Fig. 2D vs. A). Neither omitting glycogen as a precipitation carrier for labeled probe (Fig. 2 E vs. A) nor lowering SSC concentrations to half during post-hybridization washes (Fig. 2 F vs. A) showed any significant improvements.

The combination of changes, i.e. to first dissolve the labeled probe in water and to increase the concentration of DTT from 200 to 800 mM in HBF, diminished the background even more than when these two changes were applied separately (Fig. 2G vs. B or C). A further improvement of the background was evident when glycogen was left out as a precipitation carrier in addition to the combined modifications mentioned above (Fig. 2 H vs. A–G).

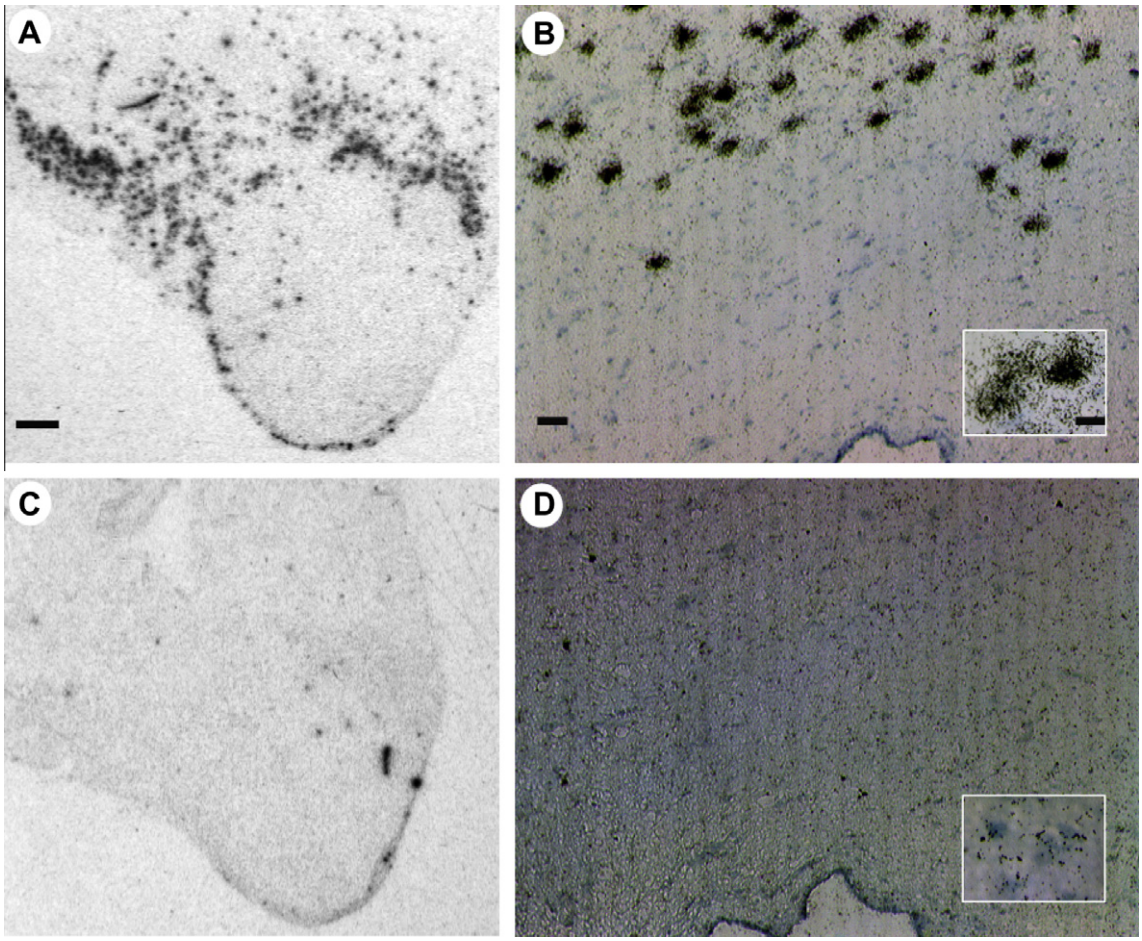

Fig. 1. Specific HDC-mRNA in situ hybridization signal was observed in the tuberomamillary nucleus of the hypothalamus (from subject #02-013) both on film autoradiograms (A) and after emulsion autoradiography with thionin-counterstaining (B), respectively, with application of HDC antisense probe. C and D are adjacent sections to A and B, respectively, incubated with the HDC sense probe following the same protocol. Insertions: higher magnification. Scale bar = 1 mm for the film autoradiograms,=50 μm for the emulsion autoradiographs; and = 12.5 μm in insertions.

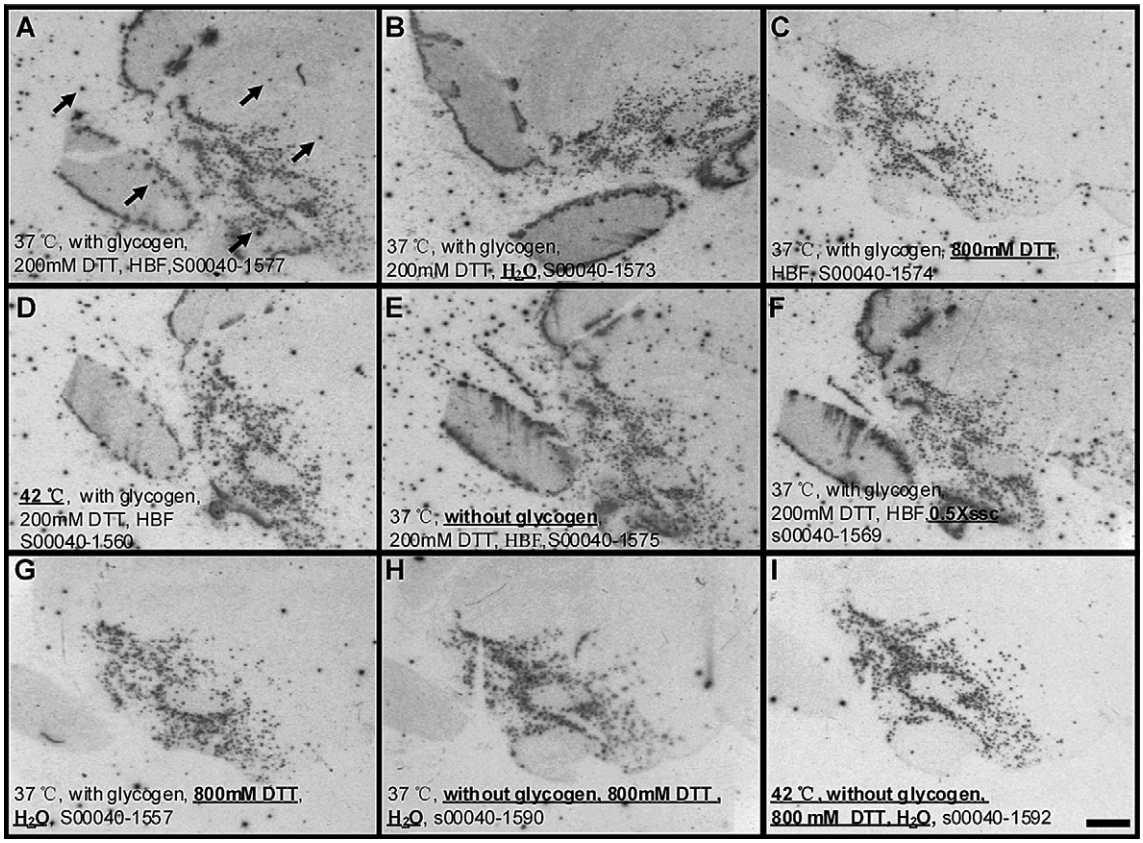

Fig. 2. Film autoradiograms (from subject #00-022) showing changes in the routine protocol which contribute significantly to a reduction of the non-specific speckles (indicated by arrows in A). (A) Routine protocol; (B) dissolving the pellet in water prior to dilution in hybridization buffer (HBF); (C) increasing the concentration of dithiothreitol (DTT) in HBF from 200 to 800 mM; (D) raising the hybridization temperature from 37 to 42 °C, with the increase of post-hybridization washing temperature from 41 to 46 °C accordingly; (E) omitting glycogen as a precipitation carrier for labeled probe; (F) lowering standard saline citrate (SSC) concentrations to half during post-hybridization washes; (G) combining changes of first dissolving the labeled probe in water and increasing the concentration of DTT from 200 to 800 mM in HBF; (H) glycogen was left out as a precipitation carrier in addition to the combined modifications of G; (I) the combination of skipping glycogen, enhancing resuspension of the labeled probe in water first, raising DTT concentration in HBF to 800 mM and increasing the hybridization temperature to 42 °C provided the best results, as characterized by a clear, specific hybridization signal and a low background. Scale bar = 1 mm. Note that the improved protocol (I) brought about significantly clearer background.

Finally, the combination of skipping glycogen, enhancing resuspension of the labeled probe first in water, raising DTT concentration in HBF to 800 mM, and increasing the hybridization temperature to 42 °C provided the best results, as characterized by a clear specific hybridization signal and a low background (Fig. 2I, Fig. 3A-b and -d).

3.3. Quantification of HDC-mRNA expression in the TMN on film

The ratio of $\text{Density}_{\text{TMN}}/\text{Density}_{\text{background}}$ from the experiment with improved protocol showed a trend to be higher than that from the routine protocol in the control group ($z = -1.955$, $p = 0.051$), and reached significance in the PD group ($z = -2.666$, $p = 0.008$) and in all the 18 pooled subjects ($z = -3.201$, $p = 0.001$) (Fig. 3B).

In the experiment with the improved protocol, the 18 subjects showed no significant correlation between the $\text{Density}_{\text{TMN}}/\text{Density}_{\text{background}}$ and age ($r = 0.23$, $p = 0.35$), fixation time ($r = -0.09$, $p = 0.72$), storage time ($r = -0.05$, $p = 0.84$) and post-mortem delay ($r = -0.24$, $p = 0.35$).

3.4. ISH of CRH-mRNA in the PVN

Fig. 4 showed ISH for the expression of CRH-mRNA in the hypothalamic PVN with the old (A) and the improved protocol (B–D). The CRH-mRNA ISH signal was located in cells within the PVN, which was evident both on film autoradiograms (Fig. 4C) and after emulsion autoradiography (Fig. 4D). Using emulsion autoradiography in combination with thionin-counterstaining, many parvocellular cells in the PVN were masked with black silver grain deposits, showing medium to heavy labeling (Fig. 4D). Specificity was also supported by the failure of the labeled sense probe to show signal in adjacent sections (Fig. 4B).

4. Discussion

The results of the present study demonstrate that the sensitivity of our previously developed ISH procedures using ^{35}S-radioactively labeled oligonucleotides can be significantly improved by a few relatively simple alterations that facilitate detection of low-to-moderate gene expression, such as HDC-mRNA in the TMN, and other targets such as CRH-mRNA in the PVN of the human hypothalamus, using formalin-fixed, paraffin-embedded archival post-mortem tissues. The modifications not only resulted in enhanced cellular localization of the HDC-mRNA signal, but also prevented the occurrence of non-specific deposits seen as speckles on film autoradiograms.

Routine formalin fixation and paraffin embedding for immunohistochemical purposes is known to induce stable crosslinks between proteins, DNA and RNA [22], which causes masking of epitopes. A number of antigen retrieval methods have been developed to improve probe penetration, including treatment with

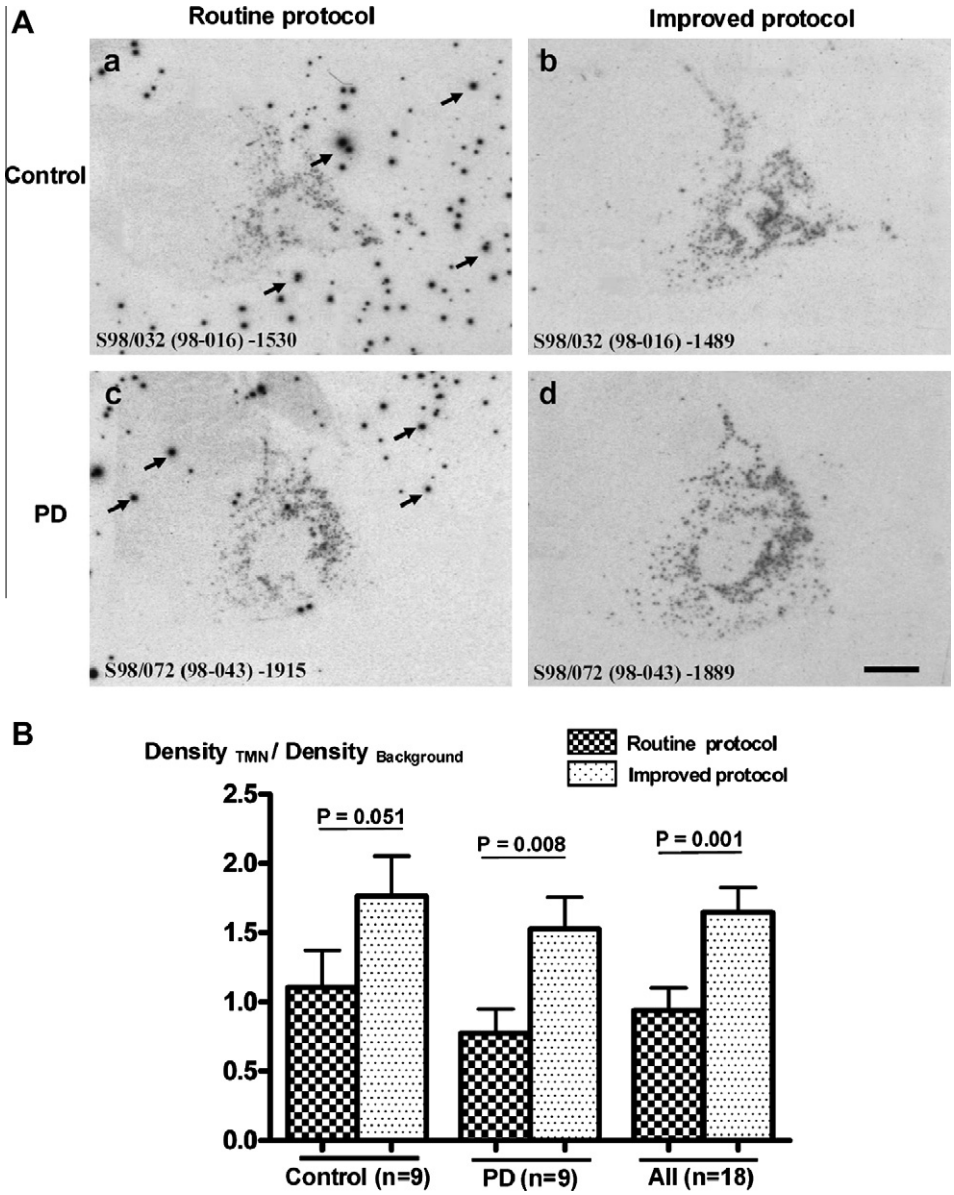

Fig. 3. (A) representative film autoradiograms of a control subject (from subject #98-016, a and b) and a PD patient (from subject #98-043, c and d), showing a clear, specific hybridization signal and lower background with the improved protocol (b and d) compared with the routine protocol (a and c). Arrows indicate the non-specific speckles. Scale bar = 1 mm. (B) the ratio of $Density_{TMN}/Density_{background}$ from the experiment with improved protocol showed a trend to be higher than that with the routine protocol in the control group ($z = -1.955$, $p = 0.051$), and reached significance in the PD group ($z = -2.666$, $p = 0.008$) and in all the 18 pooled subjects ($z = -3.201$, $p = 0.001$).

denaturing agents, heating and enzymatic digestion (reviewed in [23]). For optimal detection of HDC-mRNA in the hypothalamic TMN, we compared several retrieval techniques, including digestion with proteinase K and heating in a microwave oven or autoclave in a number of buffers at different pH values. Autoclaving the sections in 0.01 M sodiumcitrate buffer pH 6.0 at 120 °C under a pressure of 1 bar for 20 min turned out to be the most adequate pretreatment in terms of specific signal intensity and background levels (data not shown). Hydrated autoclaving has been reported previously to enable the use of formalin-fixed, paraffin-embedded sections for ISH in rat [24] and human brain [25,26] and even allowed quantitative assessment of mRNA abundance [26].

One of the improvements made to the routine ISH protocol concerned the resuspension of the labeled probe prior to hybridization. Following the tailing reaction, the labeled probe is purified by ethanol precipitation and after centrifugation it is contained within the pellet while the unincorporated ^{35}S-dATP remains in the supernatant. Using the routine protocol, we noticed that dissolving the pellet immediately in HBF for hybridization on sections often resulted in incomplete resuspension with a number of small pellet particles persisting, no matter how vigorous the mixing had been. We argued that the non-specific deposits observed on autoradiographic film may be caused by this incomplete pellet resuspension due to the high viscosity of HBF. Dissolving the pellet in sterile water to enhance resuspension prior to dilution in HBF indeed clearly suppressed the occurrence of the speckles.

Furthermore, to facilitate pellet resuspension, we tested the possibility of omitting glycogen as a carrier of precipitation. Glycogen is widely used to increase recovery of nucleic acids by alcohol precipitation and in case of low amounts of DNA it permits easy handling by visualization of the pellet after centrifugation. However, in our case we suspected glycogen to contribute to inefficient resuspension by forming a compact, dense pellet. Surprisingly, omission of glycogen by itself showed none or only marginal

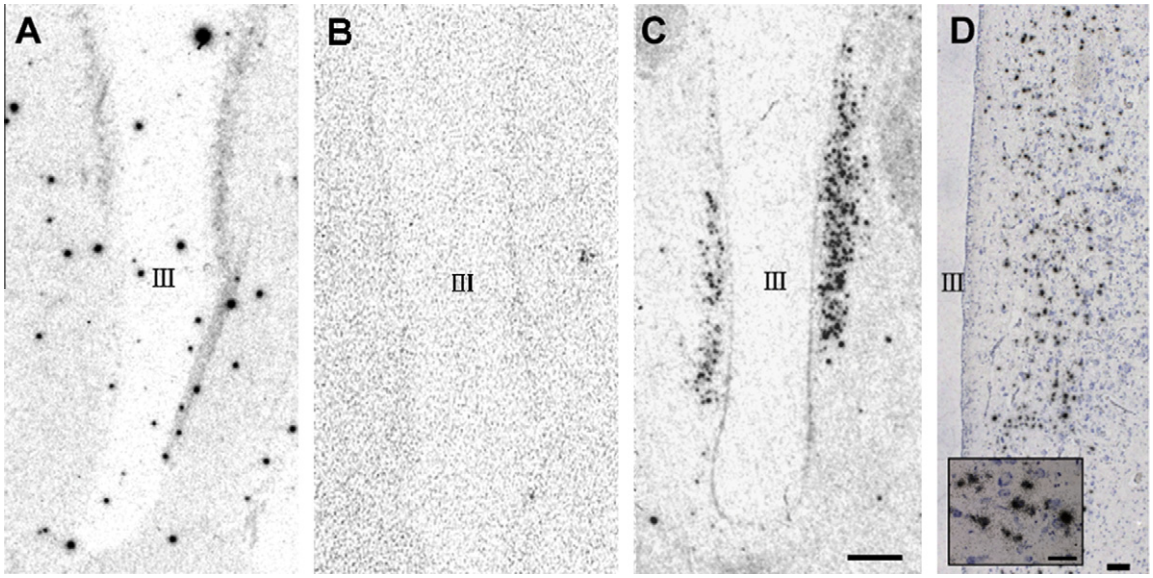

Fig. 4. CRH-mRNA in situ hybridization (ISH) signal in the human hypothalamic paraventricular nucleus (PVN) using the routine (A) and the improved protocol (B–D). CRH-mRNA ISH signal was observed on both film autoradiograms (C) and after emulsion autoradiography with thionin-counterstaining of the PVN area (D) following application of a CRH antisense probe. Incubating a section with a CRH sense probe did not reveal any signal (B). Please note the significantly clearer, specific hybridization signal and lower non-specific background in C and D compared to A. Insertion: larger magnification. III: the third ventricle. Scale bar = 1 mm for the film autoradiograms;=100 μm in for the emulsion autoradiographs, and = 50 μm in insertion.

beneficial effects on the background. Nevertheless, the combination of leaving out glycogen, resuspending the labeled probe first in water, increasing DTT concentration in HBF to 800 mM and elevating the hybridization temperature to 42 °C provided the best result, characterized by clear labeling of TMN cells and a low background with markedly fewer speckles.

Although DTT is known to reduce non-specific labeling, the underlying mechanism is poorly understood. As a reducing agent it is thought to prevent the formation of disulfide bonds between sulfur atoms and to stabilize ^{35}S probes by keeping them from oxidizing [27]. By blocking the formation of disulfide bridges within the tissue during hybridization, DTT hinders interactions between the phosphorothinoate moiety of ^{35}S and these endogenous bridges, thereby suppressing background labeling [28]. In agreement with this, increased DTT concentrations of 750–1000 mM in HBF have been reported to represent an efficient tool to reduce the high background observed with ^{35}S-labeled complementary RNA probes in frozen rat tissue sections [29]. Similarly, our results show that high concentrations of DTT in HBF have a favorable effect on the background in formalin-fixed paraffin-embedded human brain tissue sections.

Raising the hybridization temperature from 37 to 42 °C and, accordingly, the post-hybridization washing temperature from 42 to 46 °C, produced a lower background labeling. The well-known rationale behind this is that a higher hybridization temperature will destabilize non-specific duplexes while only the most homologous sequences will hybridize, thus lowering non-specific hybridization. It should be noted that once the melting point of the double-stranded complex is exceeded hybridization might no longer take place, and the duplexes formed between the probe and the target mRNA might dissociate, leading to inefficient hybridization. However, in the present study specific the HDC-mRNA ISH signal in the TMN was barely affected by the increase in temperature.

Quantification of HDC-mRNA ISH signal on sections from control and PD subjects confirmed the stability and validity of the improved protocol, as shown by significantly increased ratios of $\text{Density}_{\text{TMN}}/\text{Density}_{\text{background}}$ in these subjects. Moreover, the ratios of $\text{Density}_{\text{TMN}}/\text{Density}_{\text{background}}$ were independent of confounding factors such as fixation time, post-mortem delay and storage time, indicating compatibility of routinely processed paraffin tissue with the employed ISH procedure and subsequent signal quantification.

The improved protocol has also given favorable results for other low-to-moderate abundant genes that previously were found to demonstrate background problems as well, such as CRH-mRNA expression in the human hypothalamic PVN, which supports the general applicability of this procedure.

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