Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3213–3215, May 1988
Neurobiology

Simultaneous demonstrations of neuropeptide Y gene expression and peptide storage in single neurons of the human brain

(cerebral cortex/hippocampus/*in situ* hybridization/complementary RNA probe/immunocytochemistry)

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Contributed by S. L. Palay, November 23, 1987

ABSTRACT **A combination of *in situ* hybridization for neuropeptide Y mRNA that used a ^{32}P-labeled complementary RNA probe and immunocytochemistry with polyclonal antibodies against neuropeptide Y were applied to human cortical brain samples to simultaneously localize neuropeptide Y and its mRNA. These two techniques allowed simultaneous identification of neuropeptide Y gene expression and peptide storage in single neurons of the human brain.**

Neuropeptide Y (NPY) is a 36-amino acid peptide (1) that is distributed widely in both the central (2–4) and the peripheral (5, 6) nervous systems. Morphological studies on the human brain have shown NPY immunoreactivity in numerous cortical interneuronal cell bodies and in a rich plexus of cortical nerve fibers (3, 4). The function of these NPY-containing neurons is under investigation, as they exhibit numerical and morphological changes in certain neurological diseases (7, 8). The regulation of NPY may include alterations in the levels of mRNA encoding for the peptide or its precursors. Steady-state levels of mRNA in single neurons can be assessed by *in situ* hybridization. Through complementary base pairing, radiolabeled polynucleotides anneal to the specific sequences of the polynucleotides in the mRNA for NPY, and the neurons containing these hybrids are detected by autoradiography.

The structure of the precursor for human NPY has been deduced by analysis of the cDNA sequence. The NPY cDNA was directionally cloned behind the bacteriophage SP6 promoter. A specific single-stranded complementary RNA probe complementary to NPY mRNA was then synthesized with SP6 polymerase. This probe was used to analyze total RNA from pheochromocytomas, adrenal medullas, and other tissues (9). With this probe to human NPY, we have identified (10) the NPY transcription sites in human cerebral cortical neurons by applying *in situ* hybridization to samples of human cerebral cortex from surgical biopsy samples and from brains obtained at autopsy after brief postmortem delays.

In the present study our goal was the simultaneous localization of NPY gene expression by *in situ* hybridization and of peptide storage by immunocytochemistry with antibodies against NPY in individual human brain cortical neurons. This goal required technical innovations because of the limitations imposed by the relatively poor visual sensitivity of the *in situ* hybridization methods, the difficulties of dealing with human tissue, and the necessity for maintaining sufficient cellular immunoreactivity for successful detection of the antigen. We attempted to enhance the reliability and sensitivity of *in situ* hybridization to demonstrate mRNA while maintaining a sufficient level of immunoreactivity to allow visualization of the peptide. Neuropeptides exist in relatively low quantities in brain neurons compared to cells containing peptide hormones, for example, in the pituitary or in the enteric nervous system. Since the steady-state level of NPY mRNA copies per neuron is also relatively low, the sensitivities of both detection methods must be high to ensure some success.

MATERIALS AND METHODS

Samples of cerebral cortex (parietal or temporal regions) were taken from neurosurgical biopsies of 18 patients and from 4 individuals postmortem. Biopsy samples of normal cerebral cortex were taken from the overlying perifocal cortex of patients who underwent surgery for removal of tumor or for intractable epilepsy. Cortical specimens varying in thickness from 2 to 4 mm were removed with the tumor as an integral part of the neurosurgical procedure. The areas immediately adjacent to the tissue used were checked in stained sections by macroscopic and microscopic examination to eliminate the possibility of tumor or alterations due to tumor invasion. The samples were either fixed immediately by immersion in a variety of fixatives or were quick-frozen in liquid nitrogen and fixed after cryostat sectioning. In addition to the biopsies, postmortem samples of temporal cortex from 4 deceased patients with no clinical manifestations of neurological disease, confirmed by gross and microscopic neuropathological examination at autopsy, were also used. The areas immediately adjacent to those used here were checked in Nissl-stained sections to confirm the absence of pathologic changes. All four brains, between 1.5 and 4.5 hr after death, were perfused through the vascular system with 4% (wt/vol) paraformaldehyde. Cryostat sections (10 μm) of the frozen material were mounted on poly(L-lysine)-coated slides (11) and dried overnight at 37°C. Vibratome sections (20 μm thick) were cut from the prefixed material and processed similarly. Sections were processed for *in situ* hybridization followed by immunocytochemistry and normal histology as explained below.

The preparation of the complementary RNA probe from the 600-base cDNA insert of pNPY3-75, the prehybridization treatment of the sections, and the hybridization procedure and blot analyses have been described elsewhere (10, 12). Hybridization was carried out with 3 ng of ^{32}P-labeled probe per section (5×10^5 cpm per section) diluted in a buffer containing 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfates, 0.30 M sodium chloride, 0.30 M sodium citrate, 0.25% bovine serum albumin, 0.25% Ficoll 400, 0.25% polyvinylpyrrolidone 360, 250 mM Tris·HCl (pH 7.5), 0.5% sodium pyrophosphate, 0.5% $NaDodSO_4$, and 250 μg of denatured salmon sperm DNA per ml. Hybridization for double-labeling studies was conducted as above with the omission of dextran sulfate from the buffer to increase the

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Abbreviation: NPY, neuropeptide Y.

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signal-to-noise ratio of the subsequent antigen–antibody reaction. After hybridization, nonspecifically bound single-stranded probe was removed by treatment with a solution containing 20 μg of RNase A per ml, 0.5 M sodium chloride, 10 mM Tris·HCl (pH 8), and 1 mM EDTA for 30 min at 37°C. Thereafter, the same sections were immediately prepared for immunocytochemistry without further dehydration. Sections were incubated in 0.5% hydrogen peroxide for up to 2 hr and in 1.0% Triton X-100 for 1 hr before antibody incubation. They were then incubated overnight in primary antiserum, diluted in 0.5% Triton X-100/1% normal goat serum to a dilution of 1:200 and in fresh antibody solution for an additional 3 hr. Subsequent processing followed the peroxidase-antiperoxidase method (13) or the procedures for immunofluorescence or immunogold silver visualization (11). The following parameters were used for the peroxidase-antiperoxidase technique: goat anti-rabbit antibody (1:200 dilution, Miles) in 0.5% Triton X-100/1% normal goat serum/isotonic phosphate-buffered saline, followed by the peroxidase-antiperoxidase reagent (1:400 dilution, Arnel), then visualization by diaminobenzidine (3). The immunofluorescence technique utilized goat anti-rabbit second antibody linked to Texas red (1:20 dilution, Miles) in 1% normal goat serum; the sections were washed extensively prior to visualization. The immunogold silver method was performed as described in ref. 11, with a goat anti-rabbit gold-labeled antibody enhanced for visualization with silver development (Janssen Pharmaceutica, Beerse, Belgium).

Anti-NPY was raised in rabbits against unconjugated porcine NPY (Amersham 1086). The antibody showed negligible crossreactivity with neuropeptide PYY and with avian pancreatic polypeptide and no crossreactivity with other peptides such as somatostatin and substance P. Incubation of tissue sections with antiserum preabsorbed with synthetic NPY completely blocked staining of neuronal processes in the hippocampus (2). Control sections were incubated with primary antiserum or with primary antiserum preabsorbed with synthetic NPY antigen (0.1 nmol/ml of diluted antiserum; Bachem Fine Chemicals, Torrance, CA).

Thereafter, sections that had been hybridized and sections that had been hybridized and treated with antibodies (double labeled) were dipped in Kodak NTB-2 emulsion (1:1 dilution in distilled water) and exposed for 5–7 days at 4°C before being developed in D19 developer (Kodak). The *in situ* hybridization autoradiograms were then dehydrated with graded ethanol solutions, and coverslips were applied.

In each sequence of experiments, four sets of sections were prepared from each specimen under examination. They were (*i*) Nissl preparations stained with cresyl violet for morphological confirmation of the location of labeled cells in the laminae of the cortex, (*ii*) *in situ* hybridization alone as a control, (*iii*) *in situ* hybridization and immunocytochemistry together (double labeling), and (*iv*) immunocytochemistry alone as a control.

Controls. A separate set of sections was treated with RNase (20 μg/ml for 1 hr at 37°C) before hybridization. A further control was carried out by omitting the probe during hybridization, although all other conditions were maintained constant. Blot analysis with human cortex poly(A)$^+$ mRNA was prepared from postmortem tissue according to standard methods (14) and has been described (10).

RESULTS

Achieving consistent double labeling with *in situ* hybridization and antibody–peptide immunocytochemistry depends upon finding an acceptable compromise among the requirement for morphological fixation of the brain tissue, the preservation of the specific mRNA despite the high levels of natural RNase, the preservation of the specific immunoreactivity of the peptide, and the simultaneous display of both in intact neurons. Brief postmortem delays and perfusion of the brains at autopsy resulted in the well-fixed samples of postmortem temporal cortex used in this study. These are likely to be among the fundamental reasons for the success of the double-labeling procedures. It has been demonstrated (10) that prefixed human brain tissue hybridized consistently, with the tissues retaining a better morphology than cortical samples rapidly frozen and then postfixed. Although the latter could produce material with neurons showing a strong hybridization signal, the morphology was poor. Several studies have demonstrated (4, 8, 13–15) the suitability of brief prefixed material for the demonstration of NPY in human cortical neurons by immunocytochemistry. The present study has identified the conditions under which both antibody binding and hybridization can be applied, particularly in prefixed material.

Hybridization, as expressed by the presence of autoradiographic silver grains clustered over neurons, was readily apparent in all the preparations in the small interneurons of the deeper layers of the cortex, particularly in laminae IV–VI, as described (10). Double labeling was seen consistently with the conventional peroxidase-antiperoxidase method, with diaminobenzidine as chromogen to localize the antigen–antibody complexes, and the description and illustrations have been taken from these results. NPY-immunoreactive neurons occur abundantly in laminae IV–VI of the cortex in all preparations studied here. These immunocytochemically labeled neurons were localized to cortical laminae as described (3, 4, 10), and their dendritic extensions and cellular size, shape, and morphology were comparable to those described.

In preparations simultaneously double labeled by *in situ* hybridization and immunocytochemistry, neurons exhibiting silver grains and the antigen–antibody complexes were also found in similar locations in the deeper layers (IV–VI) of the cerebral cortex. Two examples of double-labeled neurons are shown in Fig. 1. The deep brown/red color resulting from the peroxidase reaction localizes the NPY antigen–antibody throughout the neuronal somata and in the primary dendrites. Overlying the cell soma, and less over the dendrites, are dense clusters of autoradiographic silver grains from the simultaneous hybridization. Neuronal nuclei, when discernible in our preparations, are not labeled by either the NPY antibody or the mRNA hybridization procedure. The incidence of double-labeled neurons is high in optimal preparations, matching quantitatively our expectations on the basis of the single-labeled control preparations made from the adjacent sections in each set of experiments.

The double-labeling procedure that we have described here overcomes a few problems that were encountered when the immunocytochemistry procedure was done before the *in situ* hybridization. In contrast to two reports, one with virus infected cell cultures (16) and another with the pituitary (15), our studies show that prior staining resulted in considerable loss of the *in situ* signal possibly because of the lengthy incubations in the primary and secondary antibodies and subsequent reactions, as well as RNase activity. The procedures that we have described in which *in situ* hybridization preceded immunocytochemistry allow a strong *in situ* hybridization signal and a relative weak antigen–antibody binding when results of the double-labeled experiments are compared to the single-labeled controls of *in situ* hybridization alone and of immunocytochemistry alone. Clearly, double-labeling experiments are compromises between results obtainable from the simultaneous juxtaposition of more than one difficult technique. However, it remains the only valid way of demonstrating the simultaneous existence of both gene expression and peptide storage in the same cell. Because of the inherent effects that *in situ* hybridization had

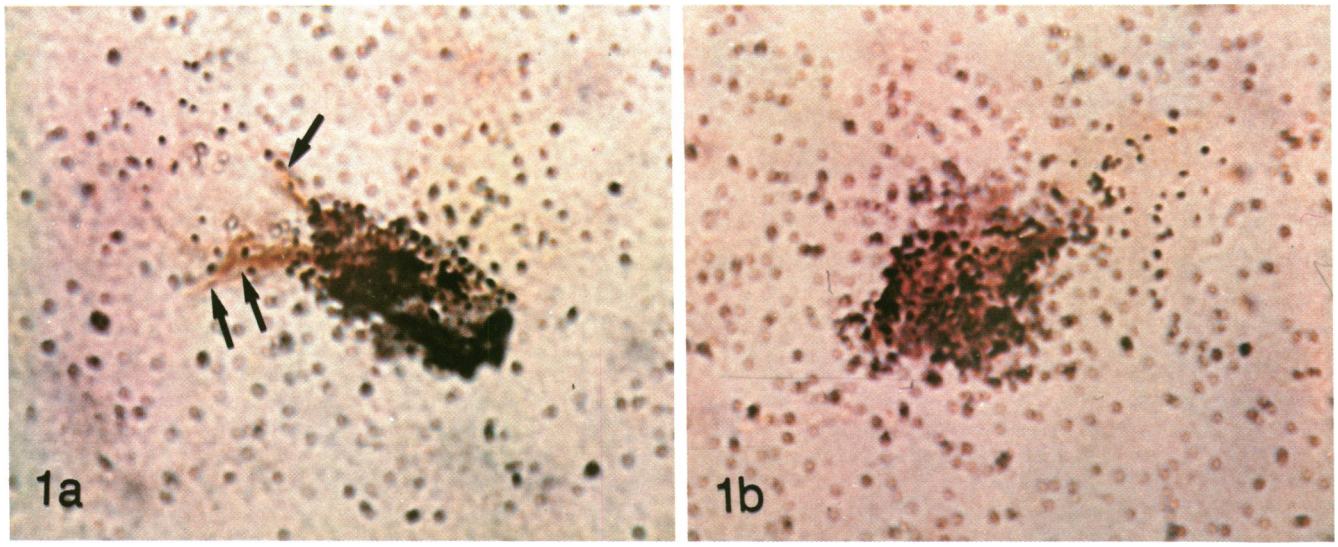

FIG. 1. Two neurons in layer VI of the human cerebral cortex labeled by immunocytochemistry (*a*) and *in situ* hybridization (*b*). The immunocytochemical label (brown) is visualized by the peroxidase anti-peroxidase reaction after incubation with antibodies directed against NPY, whereas the autoradiographic silver grain clusters concurrently over the same cell somata demonstrate hybridization sites labeled by the ^{32}P-labeled ribosomal probe for the NPY gene. Note that the dendrites (arrows) labeled by the antibodies against the peptide (*a*) are not labeled with autoradiographic silver grains (*b*). (×1000.)

upon the immunocytochemical staining, it is difficult to quantitatively assess immunocytochemical staining from double-labeling work. In many preparations all cells with grain clusters from the *in situ* signal had NPY immunoreactivity. However, several preparations with neurons labeled by *in situ* hybridization had only weak or no NPY immunoreactivities. Rather than concluding with the possible biological explanation—that some neurons that express the NPY gene do not store the peptide—we prefer to conclude that technical difficulties can impair consistent demonstrations of NPY immunoreactivity.

In the control preparations treated with RNase prior to hybridization, no hybridized cells could be found. Similar results were found when the probe was omitted during the hybridization step. RNA blot hybridization analysis with a human NPY cDNA probe detected a single species of ≈800 bases as reported (10). These results of blot analysis are consistent with reports on several RNA preparations, including those from human pheochromocytoma (9), a tissue known to contain significant NPY immunoreactivity. RNAs prepared from other tissue sources do not show significant hybridization to the complementary RNA probe. In control preparations for NPY antibody specificity, incubation of adjacent sections in antibody solutions preadsorbed with NPY demonstrated no reactive neurons or fibers.

DISCUSSION

We have described the methods by which NPY gene transcription and NPY storage can be simultaneously demonstrated in single human cortical neurons by using *in situ* hybridization with complementary RNA probes and immunocytochemistry. The combination of those two methods has unequivocally demonstrated that single human intracortical neurons transcribe NPY mRNA and also store the peptide. This demonstration answers a question often raised against demonstrations of neurons containing peptide immunoreactivity. That is, do these peptide-containing neurons synthesize the neuroactive peptide in question, or is the presence of the peptide purely incidental? We envision that further applications of the methodological advances reported here will include investigations into the question of genetic transcription of multiple neuroactive substances in single neurons.

We thank U. Haesler, C. Adams, and M. Dunkley for excellent technical assistance. This work was supported by US AFOSR Grant 86-0176, Amersham International, Astra Alab, Cambridge Research Biotechnicals Limited, and the Wellcome Trust. Q.H. was supported by the Gulf University of Bahrain and an Overseas Research Award.

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