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Improved method for combination of immunocytochemistry and Nissl staining

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Abstract

Nissl-staining is a widely used method to study morphology and pathology of neural tissue. After standard immunocytochemistry, the Nissl-staining labels only the nucleus of neurons and the characteristic staining of the neuronal perikarya is absent or very weak. We hypothesized that the RNA degradation during the immunocytochemical treatment results in the loss of cytoplasmic staining with Nissl-dyes. To test this hypothesis, we used RNase-free conditions for all steps of immunostaining. To further prevent the RNA-degradation by RNase contaminations, the RNase inhibitor heparin was added to all antibody-containing solutions. The efficiency of Nissl-staining after standard and RNase-free double-labeling immunocytochemistry was compared using antibodies against c-Fos and neuropeptide Y (NPY) on tissues of rats refed after three days of fasting. After standard immunocytochemistry, the Nissl-staining labeled the nuclei of neurons and only very faintly the cytoplasm of these cells. The RNase-free treatment did not alter the distribution of immunoreaction signal, but preserved the staining of neuronal perikarya by the Nissl-dyes. In conclusion, the RNase-free conditions during immunocytochemistry, allows the labeling of neuronal perikarya by Nissl-dyes. The described method facilitates the mapping of immunocytochemical signals and makes possible the light microscopic examination of the innervation of neurons identified by their nuclear protein content.

Keywords

immunocytochemistry; Nissl-staining; counter-staining; RNase-free conditions

Introduction

The histological method invented by Nissl (Nissl, 1894) is widely used by neuroanatomists and pathologists to study the morphology and pathology (Fujita et al., 2008; Pilati et al.,

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2008; Robak, 2002) of neurons and also to understand the cytoarchitectony (Kulesza, 2008; Lorenzo et al., 2008) of different brain areas. The Nissl-staining method is based on the interaction of basic dyes such as cresyl violet, thionine, toluidin blue, methylen blue or anylin with the nucleic acid content of cells. These dyes can bind to the DNA content of the cell nuclei, but also to the RNA (Scott and Willett, 1966) that is highly concentrated in rough endoplasmic reticulum and ribosomes (Nissl substance) in the cytoplasm. Since neurons are very active protein synthesizing cells, the cytoplasm of these cells contain high concentration of rough endoplasmic reticulum (Knowles et al., 1996; Kosik and Krichevsky, 2002). Due to this special characteristic of neurons, the Nissl staining can specifically stain the cytoplasm of neurons without recognizing the perikarya of other cellular elements in the brain. This is an advantage of Nissl staining compared to other counterstaining methods, such as neutral red stining that also labels astrocytes (Gonthier et al., 2004). Therefore, Nissl-staining is very useful method to study the pathology of neurons, and understand the cytoarchitectony of different brain areas.

Nissl staining is also often used to counterstain sections after immunocytochemical detection of specific molecules in the brain to facilitate the localization and mapping of the labeled cell populations. Unfortunately, however, Nissl staining very frequently labels only the cell nuclei after the immunocytochemical procedure that makes the counterstaining less valuable (Fekete et al., 2004; Krout et al., 2002; Lefler et al., 2008).

The fact, that only the labeling of cytoplasm is lost after immunocytochemistry but the basic dyes still stain the cell nuclei, very similarly than after *in situ* hybridization, when RNase treatment is used (Kiss et al., 2007), suggested that during the immunocytochemical treatments the RNA content of neurons may be degraded. Therefore, we have hypothesized that RNase free conditions during immunocytochemistry may improve the counterstaining with basic dyes.

To test this hypothesis, we have compared the counterstaining after standard and RNase free immunocytochemical procedures.

Materials and Methods

Animals

Three adult male Wistar rats weighing 310g were used in this study. The animals were housed in cages under standard environmental conditions (light between 6–18h, temperature 22°C, free access to water). The animals were fasted for 3 days, then, refed 2 hours before perfusion. All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine of the Hungarian Academy of Sciences.

Tissue preparation

Three rats were deeply anesthetized i.p. with ketaminexylazine (ketamine: 50 mg/kg; xylazine: 10 mg/kg body weight) and were perfused by intracardiac perfusion with 20ml 0.01M PBS (pH=7.4) followed by 150ml 4% paraformaldehyde in 0.1M PB (pH=7.4). The brains were removed and postfixed in 4% paraformaldehyde in 0.1M PB at room temperature for 2 hours. The brains were cryoprotected in 20% sucrose in PBS at 4°C overnight, then frozen on dry ice. 25-µm-thick coronal sections were cut by freezing microtome, collected in cryoprotective solution (30% ethylene-glycol; 25% glycerol; 0.05 M phosphate buffer) and stored at –20°C until used.

All solutions that were used for fixation and section storage were RNase-free. The MilliQ-water that was used for the solutions and the 0.1M PB and PBS stock solutions were treated with diethylpyrocarbonate (0.2µl cc. diethylpyrocarbonate /ml) overnight at room temperature and then autoclaved.

Standard double-labeling immunocytochemistry

The sections were treated with 0.5% Triton X-100 and 0.5% H₂O₂ in 0.01M PBS (pH=7.4) for 15 min. To reduce the nonspecific antibody binding, the sections were treated with 2% normal horse serum in PBS for 15 min. Then, the sections were incubated in rabbit antiserum against c-Fos (at 1:30,000 dilution, Ab-5, Calbiochem, San Diego, CA, USA) in PBS containing 2% normal horse serum, 0.2% Kodak Photo-Flo and 0.2% sodium azide for 2 day at 4°C. Kodak Photo-Flo was used as a detergent. Then, the sections were washed in PBS and incubated in biotinylated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, PA, USA) at 1:500 dilution for 1.5 hour at room temperature, followed by an incubation in Avidin-Biotin-Peroxidase (Vector Laboratories, Inc., CA, USA) complex at 1:1,000 dilution in 0.05M Tris buffer (TB) (pH=7.6) for 1 hour. The immunolabeling was visualized by 0.05% DAB/0.15% Ni-ammonium-sulfate/0.005% H₂O₂ in 0.05 M TB. Then the sections were incubated in sheep anti-neuropeptide Y serum (at 1:100,000 dilution, gift from Dr. István Merchenthaler, University of Maryland, School of Medicine, Baltimore, USA) in TB containing 2% normal horse serum, 0.2% Kodak Photo-Flo and 0.2% sodium-azide for 1 day at 4°C. After that, the sections were rinsed in PBS and incubated in biotinylated donkey anti-sheep serum at 1:500 in TB containing 2% normal horse serum and 0.2% sodium azide. Then the sections were incubated in ABC at 1:1,000 dilution in 0.05M TB (pH=7.6) for 1 hour. The immunolabeling was visualized by 0.025% DAB/0.0036% H₂O in TB.

RNase free double-labeling immunocytochemistry

For RNase-free immunocytochemistry, the sections were treated very similarly than for standard immunocytochemistry but with the following modifications. All solutions were prepared with MilliQ water treated with diethylpyrocarbonate (DEPC, 0.2µl/ml) (Wiener et al., 1972) overnight and autoclaved. PBS in stock solution was also treated with DEPC (0.2µl/ml) overnight at room temperature and autoclaved. Antibodies were dissolved in the following solutions: 1% bovine serum albumin, 0.2% sodium azide and 15 U/ml heparin in 0.01M PBS or 0.01M TB. Heparin was used to inhibit the RNase activity of the antisera (Ukita et al., 1962). The primary antibodies were used in the following concentrations: rabbit antiserum against c-Fos at 1:15,000 dilution and sheep anti-neuropeptide Y serum at 1:50,000 dilution.

Counterstaining

Sections mounted on gelatine-coated slides were dehydrated with ascending series of ethanol, treated with xylene for 5 min and rehydrated in descending series of ethanol and in MilliQ water. Then, the sections were treated with 1 % cresyl violet (Sigma) solution for 3 min followed by differentiation in acetic acid in 100% ethanol (at 1:50,000 dilution) for 5 sec. Then, the sections were dehydrated in ascending series of ethanol, treated with xylene and coverslipped using DPX Mounting medium (Sigma-Aldrich, Inc., USA).

Image analyses

Photographs were taken with AxioImager M1 microscope (Carl Zeis, Inc., Jena, Germany) using AxioVision software. Brightness and contrast were adjusted with Adobe Photoshop software.

Results

Effects of RNase free immunocytochemical method on the c-Fos immunostaining in the hypothalamus after refeeding

Two hours after refeeding, the hypothalamic nuclei including the ventral parvocellular subdivision of the hypothalamic paraventricular nucleus (vPVN) showed identical distribution pattern of c-Fos immunoreactive (IR) neurons as it was described earlier (Singru et al.,

2007). The RNase-free treatment did not alter the distribution of c-Fos immunoreactivity (Fig. 1AD).

Analysis of the juxtaposition of NPY-IR varicosities and c-Fos-IR neurons in the vPVN after refeeding using standard immunocytochemical method combined with Nissl counterstaining

Nissl-counterstaining after standard immunocytochemistry resulted in labeling of cell nuclei, but stained only very faintly the neuronal perikarya (Fig. 1A, C, E). Numerous NPY-IR fibers were detected in the vPVN around the c-Fos containing neurons (Fig. 1A, C, E). However, due to the very faint cytoplasmic staining, the extent of cytoplasm could not be detected (Fig. 1C, E), and therefore, it was not possible to determine the number of c-Fos-IR neurons contacted by NPY-IR varicosities.

Analysis of the juxtaposition of NPY-IR varicosities and c-Fos-IR neurons in the vPVN after refeeding using RNase free immunocytochemical method combined with Nissl counterstaining

After RNase-free immunocytochemistry, cresyl violet dye stained not only the cell nuclei but also labeled the cytoplasm of neurons (Fig. 1B, D, F). This cytoplasmic staining facilitated the identification of the different subdivisions of the PVN and the correct location of labeled cell populations (Fig. 1B). In addition, the clear labeling of cytoplasm made the cell borders visible (Fig. 1F). C-Fos-IR neurons in the vPVN were contacted by numerous NPY-IR fibers (Fig. 1D, F). By quantification of the contacts, we have observed that $84.99 \pm 2.35\%$ of c-Fos-IR neurons were contacted by NPY-IR varicosities.

Discussion

The Nissl staining is a very useful histological method to facilitate the identification of cell populations in histological preparations. Unfortunately, however, when Nissl-staining is combined with immunocytochemistry, the basic dyes stain primarily the cell nuclei with only faint labeling of cytoplasm of neurons. Since, the basic dyes bind to the nucleic acid content of cells and RNase treatment during the posthybridization step of *in situ* hybridization also results in the loss of the cytoplasmic staining with the basic dyes (Kiss et al., 2007), we hypothesized that the RNase contamination of the solutions used for immunocytochemistry may be the reason of the lack of the labeling of Nissl bodies with basic dyes. This hypothesis is also supported by data showing that immunocytochemistry results in damage of RNA in samples processed for microarray analyses (Wang et al., 2006).

To test this hypothesis, we have used DEPC-treated, autoclaved buffers and RNase free glassware for all steps of immunocytochemistry. To further prevent the potential RNase contaminations, the RNase inhibitor heparin was added to all antibody containing solutions. The RNase free conditions slightly decreased the sensitivity of the immunocytochemistry, but it could be compensated by an increase of the concentration of the primary antibodies. This effect of RNase free conditions is probably due to the protease activity of heparin. The distribution of the immunoreaction products was not influenced by the treatment. The RNase free conditions, however, markedly changed the result of the counterstaining. The Nissl staining strongly labeled the cytoplasm of the neurons and also resulted in a slightly more intense nuclear labeling.

The combination of RNase free immunocytochemistry and Nissl staining can be a very useful approach when precise localization of immunostained neuronal elements is necessary: mapping the localization of new peptides, proteins, identifying regions where anterogradely

or retrogradely-labeled structures (Quinn et al., 1995) can be found or mapping the localization of activated neurons using c-Fos as a marker (Herrera and Robertson, 1996).

In addition, the strong cytoplasmic labeling of neurons with Nissl staining can be helpful, when the innervation of a cell group is studied at light microscopic level and the neuronal population is identified based on a marker that is localized in the nucleus of cells, like nuclear receptors and transcription factors. As an example, we show that it is very difficult to determine at light microscopic level whether the NPY-IR varicosities are juxtaposed to the surface of the c-Fos-IR neurons in the ventral parvocellular subdivision of the PVN when the cytoplasm of cells is not or very faintly stained. Due to the lack of the cytoplasmic staining, the borders of the c-Fos expressing cells can not be visualized. In contrast, when RNase free double-labeling immunocytochemistry is combined with Nissl staining, the brown DAB, the dark blue Ni-DAB and the purple Nissl staining that strongly labels the cytoplasm of neurons can be easily distinguished. Therefore, the juxtaposition of NPY-IR varicosities to the c-Fos-IR neurons in the vPVN can be easily detected and quantified. By this method we observed that in the vPVN $84.99 \pm 2.35\%$ of the c-Fos expressing neurons are contacted by NPY-IR fibers.

In summary, we conclude that when standard immunocytochemistry is combined with Nissl-staining, the loss of the cytoplasmic RNA content of neurons results in the lack of cytoplasmic labeling with the Nissl staining. In contrast, combination of RNase free immunocytochemistry with Nissl staining preserves the RNA content of neurons and results in a strong cytoplasmic counterstaining that can facilitate mapping of immunostained neurons or the light microscopic examination of the innervation of cells characterized by their nuclear protein content.

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Figure 1.

Combination of double-labeling immunocytochemistry for NPY (brown) and c-Fos with Nissl-staining (violet) using standard (A, C, E) and RNase free (B, D, F) immunocytochemical techniques on sections containing the hypothalamic paraventricular nucleus (PVN) of rats refed after three day fasting. Low and medium power micrographs illustrate the distribution of NPY- and c-Fos-IR elements in the PVN (A, B) and in the ventral parvocellular part of the PVN (C–F), respectively. The distribution of the immunoreaction signals is identical on section immunostained with standard (A, C) or RNase free (B, D) method, however, the Nissl staining resulted in only nuclear or pale cytoplasmic labeling after the standard immunocytochemistry (A, C) while strong cytoplasmic Nissl-staining is observed after RNase free

immunocytochemistry (B, D). High power micrographs (E, F) illustrate the the relationship of NPY-IR varicosities and the c-Fos-IR neurons in the ventral parvocellular subdivision of the PVN. Although, NPY-IR varicosities surround the c-Fos-IR neurons of both images (E, F), the cytoplasmic border of c-Fos-IR neurons and the juxtaposition of NPY-IR varicosities (arrows) and the c-Fos-IR neurons can be recognized only on the preparations stained with RNase free immunocytochemistry (F). Scale bar on B = 100 μ m corresponds to A and B, scale bar on D = 100 μ m corresponds to C and D, scale bar on F = 20 μ m corresponds to E and F.