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IN SITU HYBRIDIZATION HISTOCHEMISTRY: A NOVEL METHOD FOR NEURONAL TISSUES STUDY

M. R. Islam[1*, 2]

[1*]Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan, and
[2]Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh
E-mail: rafiqah77@yahoo.com

ABSTRACT

The aim of the present study to explore the convenient method of in situ hybridization histochemistry for neuronal tissues studies. The glutamate receptor 1 (GluR1) 35S labeled oligonucleotide probe was used for in situ hybridization histochemistry. The glutamate receptor 1 mRNA expression was found abundantly in the dentate gyrus, Ammon`s horn, and subiculum of the rat hippocampus. In comparison to immunohistochemistry as well as use several types of probes like double strand DNA probe, single strand DNA probe, and RNA probe, the most convenient is oligonucleotide probe for the rapid assessment of the expression of a cloned ion channel by in situ hybridization histochemistry.

Key words: Oligonucleotide probe, autoradiogram, hippocampus, rat

INTRODUCTION

In situ hybridization, also referred to a hybridization histochemistry was introduced in 1969 (Buongiorno-Nardelli and Amaldi, 1969; John *et al*., 1969). In situ hybridization is method of localizing and detecting specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest (Hofler, 1990). In situ hybridization technique and its application have been carried out in all different tissues, but particularly useful in neuroscience where the tight regulation of gene transcription is vital to the operation of the brain (Tecott *et al*., 1987; Sato and Tohayama, 1998). Using this technique, it is possible to locate gene expression to specific cell types in specific regions and observe how changes in this distribution occur throughout the development and correlate with the behavioral manipulations. Although immunohistochemistry is a powerful tool to detect the exact localization of protein but it is sometimes time-consuming to produce a good antibody. Therefore, the most convenient method for the rapid assessment of the expression of a cloned ion channel could be in situ hybridization histochemistry (Nilaver, 1986). If there is plasmid vector including the corresponding cDNA, in situ hybridization histochemistry with cRNA probe can be immediately performed. Even if the cDNA are not available, synthesizing oligo probe or sub-cloned PCR products enable in situ hybridization histochemistry with oligonucleotide probe and cRNA probe (Sato and Tohayama, 1998). Thus, this paper was performed to describe the easiest way of in situ hybridization histochemistry using oligonucleotide probe for neuronal tissues.

MATERIALS AND METHODS

The present study was conducted at the Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University, Japan, during the period from July 2006 to June 2007.

General considerations

All buffers and equipments used for in situ hybridization experiments were autoclaved and sterilized by dry heater to avoid RNase contamination and keep mRNA intact. All procedures were performed with gloves.

Basic solutions

20x SSC: Nacl of 175.3 g and sodium citrate of 88.2 g were dissolved in 800 ml of distilled water. After adjustment of pH to 7.0 and volume to 1000ml were sterilized by autoclaving.

1.2M phosphate buffer (pH7.4): NaH2PO4-2H2O of 3.56 g and Na2HPO4-12H2O of 34.8 g were dissolved in 100 ml distilled water and sterilized by autoclaving.

0.2M phosphate buffer (pH7.4): NaH2PO4-2H2O of 5.93 g and Na2HPO4-12H2O of 58 g were dissolved in 1000 ml distilled water and sterilized.

1M dithiothreitol (DTT): Dithiothreitol of 154 mg was mixed in 1 ml disterilized distilled water (DDW) and kept at -20 ˚C.

Coating of micro slides with 3-aminopropyltriethoxysilane

The micro slides were washed with detergent and dried completely. Then the slides were immersed into the acetone followed by acetone containing 2% 3-aminopropyltriethoxysilane, acetone and distilled water for 5 minutes in each step. After drying, slides were sterilized at 180 ˚C for 4 h and kept at room temperature until use.

Animals and tissue preparation

Four adult rats (weighing 250 to 270g) were used in the present study. The animal handling procedures were approved by the Animal Experimental Committee of the Faculty of Applied Biological Sciences, Gifu University. The animals were anesthetized and sacrificed by sodium pentobarbital (50 mg/kg), and fresh brains were quickly removed and immediately frozen on powdered dry ice. The coronal sections at hippocampus region were cut at 30 µm on a cryostat, thaw-mounted onto the 3-aminopropyltriethoxysilane coated slides and stored at -30°C until use.

Oligonucleotide probes

The 45 mer antisense and sense oligo cDNA probes of glutamate receptor 1 (GluR1) were designed based on the rat GluR1 cDNA sequence (Boulter *et al*., 1990) and were synthesized commercially (Rikaken, Nagoya, Japan). The sequence of the GluR1 antisense is:

5’-GTCACTGGTTGTCTGGTCTCGTCCCTCTTCAAACTCTTCGCTGTG-3’, and the complementary sequence of antisense probe used as sense probe. The probes were labeled at 3’-end with [35S] dATP (46.25 TBq/mmol; PerkinElmer Life Science, Waltham, MA, USA) using terminal deoxynucleotidyl transferase (Takara, Tokyo, Japan) to obtain a specific activity of approximately 1-2 × 109 d.p.m/µg.

In situ hybridization

The slide-mounted sections being warmed to room temperature were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min (all steps were performed at room temperature unless otherwise indicated), rinsed three times (5 min each) in 4X standard saline citrate (SSC; pH 7.4; 1X SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate), and dehydrated through a graded ethanol series (70-100%). Sections were then defatted with chloroform for 3 min, and immersed in 100% ethanol (twice for 5 min each time) before being subjected to hybridization. Then the hybridization was performed by incubating the sections with the following buffer at 41°C overnight: 4X SSC, 50% deionized formamide, 0.12M phosphate buffer (pH 7.4), 1% Denhardt’s solution (Nacalai, Kyoto, Japan), 0.025% yeast tRNA (Roche, Mannheim, Germany), 10% dextran sulfate (Nacalai, Kyoto, Japan). The buffer contained probes labeled with [35S] dATP (46.25 TBq/mmol; PerkinElmer Life Science, Waltham, MA, USA; approximately 1-2 × 107 d.p.m/ml, 0.3 ml/slide). After hybridization, sections were rinsed in 1X SSC (pH 7.4) for 10 min followed by rinsing three times in 1X SSC at 55°C for 20 min, dehydrated through a graded ethanol series (70 to 100%), and sections were exposed to x-ray film (Fuji Medical X-Ray Film, Tokyo, Japan) for 7 days. After film autoradiograms, sections were coated with NTB-2 emulsion (Eastman Kodak Company, Rochester, NY, USA) diluted 1:1 with distilled water and exposed at 4°C for 4 weeks in a tightly sealed dark box. After being developed in D-19 developer (Eastman Kodak Company, Rochester, NY, USA), the sections were fixed, and washed with tap water and dehydrated. Some sections were counterstained with 0.1% cresyl violet to allow morphological identification.

Control studies

The complementary sequence of antisense probe was used as a sense probe for negative control in this experiment.

RESULTS AND DISCUSSION

The x-ray film autoradiograms revealed the GluR1 mRNA expression pattern in the hippocampal region of the rat brain (Fig. 1A). The emulsion-coated developed sections were used to identify the cellular localization of the GluR1 mRNA, and sections were observed two different images, bright-field image and dark-field image. Abundant GluR1 mRNA expression was found at the dentate gyrus (DG) and Ammon's horn (CA1 to CA3 regions), and subiculum of the rat hippocampus at the bright field photomicrographs (Fig. 1B) that followed the dark field photomicrographs (Fig. 1C). The GluR1 mRNA expression was observed in the neurons of the hippocampus (Fig. 1D).

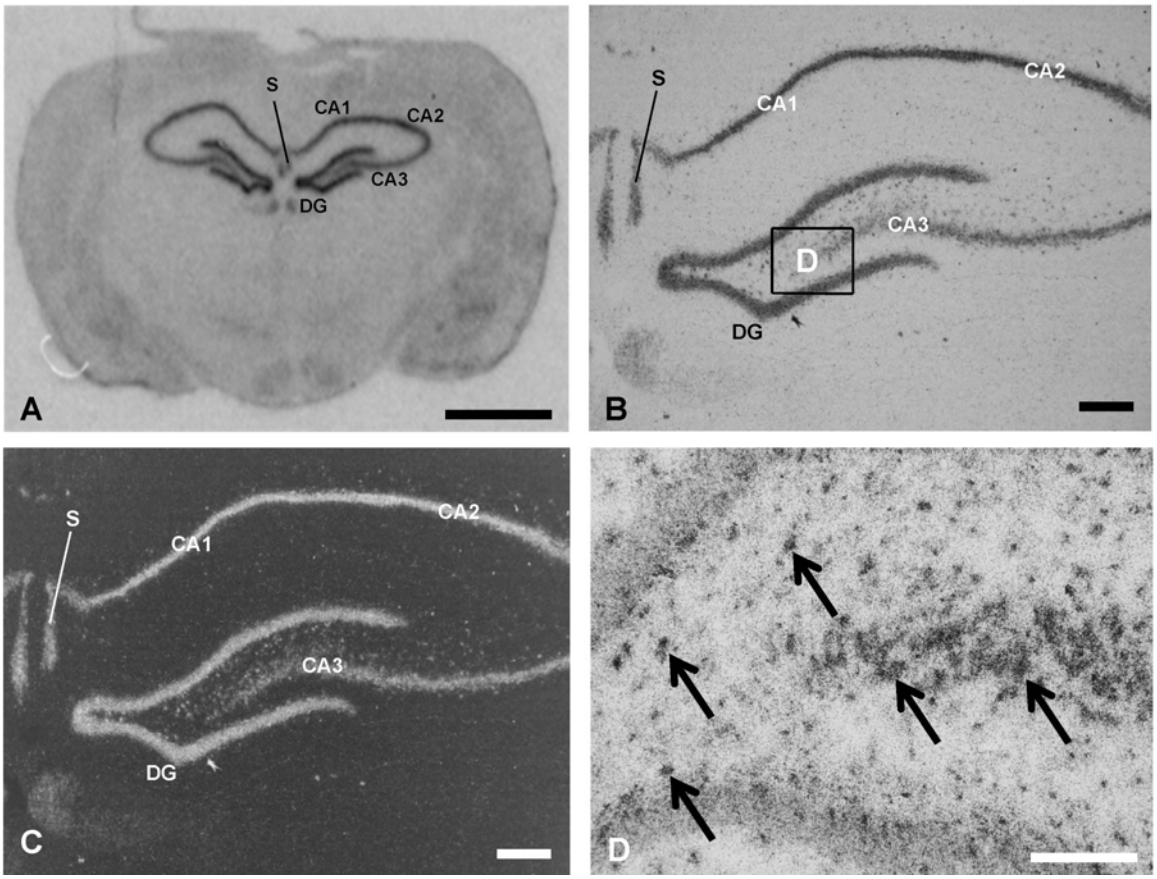

Fig. 1A-D. The x-ray film autoradiogram and photomicrographs showing GluR1 mRNA expression in the hippocampus (dentate gyrus, DG and Ammon's horn - CA1 to CA3 regions, and subiculum, S) of the rat brain. A: X-ray film autoradiogram of rat brain section. B: Photomicrograph of emulsion-coated section in bright field image. C: Photomicrograph of emulsion-coated section in dark field image. D: Enlargement of a box B. GluR1 mRNA expressed in the hippocampal neurons (arrows). Scale bars in A = 2mm, B and C = 50µm, and D = 200µm.

The identical hybridization conditions the sense probe did not show any expression in the brain tissue. The similar pattern mRNA expression and signals intensity are also found in these regions using cRNA probe in rat brain (Rogers *et al*., 1991). In situ hybridization is a unique and powerful technique and has several advantages over the immunohistochemistry especially in the anatomical localization. The nature of the immunohistochemistry procedure makes it non-quantifiable and is frequently fettered with both false negative and positive results as well as deleterious effects of fixatives in altering tissue antigenicity (Nilaver, 1986). While in situ hybridization cannot provide false information of mRNA expression and has no fixative effects on frozen tissues (Sato and Tohayama, 1998). In situ hybridization can provide converging evidence to support functional interpretations of the data, and in situ hybridization is inherently a correlative measure. Like any correlation measure, causative interpretations are speculative. In order to substantiate causative interpretations, careful manipulations must be performed. One such manipulation that has been used recently is the local injection of antisense oligodeoxynucleotides into the brain region to bind mRNA and inhibit protein expression (Guzowski *et al*., 2000). This allows causal and functional hypotheses generated from in situ hybridization data to be tested directly. Despite the advantages of in situ hybridization histochemistry, it is extremely important that the proper control studies are performed to show that the labeling is due to hybridization of the target rather than non-specific labeling.

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