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Simultaneous Detection of OPRM1 Protein and Its Message in Rat Brain Using Immunohistochemistry and RNAscope® *In Situ* Hybridization Technology

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Introduction

The μ Opioid Receptor (OPRM1), also known as MOR and MOR1, is a 60–70 kDa variably glycosylated G protein-coupled receptor that mediates many of the therapeutic effects of alkaloid and peptide opioids, including morphine, as well as their side-effects such as tolerance and dependence. OPRM1 is primarily expressed on neurons in the brain, spinal cord, and gastrointestinal tract, but can also be found on immune cells. The OPRM1 gene undergoes extensive alternative splicing, producing multiple splice variants that can be classified into three groups based on structure: full-length carboxy terminal variants with 7 transmembrane (TM) domains, truncated 6-TM variants, and single TM variants. These splice variants have been shown to exhibit different expression patterns and regional specificity within the central nervous system (CNS), and mediate the different pharmacological activities of opioids.

Over the past few decades, RNA has been recognized as a potential marker for various biological states. With recent improvements in specificity and sensitivity, *in situ* hybridization (ISH) has become increasingly important in elucidating the role of RNA as a biomarker. This technique has a number of advantages when compared with other transcriptome technologies such as microarrays, RNA sequencing, and PCR. While these later methods provide only quantitative data, ISH allows the researcher to study RNA expression within a tissue, which provides morphological and spatial contexts to the target. The RNAscope® ISH technology from Advanced Cell Diagnostics, a Bio-Techne Brand, is particularly advantageous because not only does it give information about RNA expression in a morphological context, it also provides quantitative data due to the nature of its single-molecule detection system.

To study the anatomical distribution of the MOR-1A splice variant among OPRM1-expressing neurons in various parts of the rat CNS, we developed a novel protocol that utilizes both the RNAscope® ISH technology and conventional fluorescence immunohistochemistry (IHC) using a rabbit monoclonal antibody directed against the first 22 N-terminal amino acids of rat OPRM1. Our results show different regions in rat CNS display different colocalization patterns of the OPRM1 protein and MOR-1A mRNA.

Materials and Methods

Animals and Tissue Preparation

Sprague Dawley rats (200 g) were anesthetized with sodium phenobarbital and transcardially perfused with a phosphate-buffered saline (PBS) solution, followed immediately by Lana's fixative and 10% sucrose in PBS. Fixed frozen rat brain, spinal cord, and dorsal root ganglia (DRG) were sectioned at 4–6 μm on a cryostat and mounted onto NewSilane adhesive coated histological slides. Animals were handled according to the Institutional Animal Care and Use Committee protocol approved by R&D Systems, Inc.

ISH Reagents

- RNAscope® ISH Multiplex Fluorescent Assay (Advanced Cell Diagnostics, Catalog # 320850)*
- RNAscope® Probe- Rn-Oprm1-C2 (Advanced Cell Diagnostics, Catalog # 410691-C2)

*Slight modifications were made to the manufacturer's protocol. See Tissue Staining Workflow for the ISH and IHC protocol that was followed.

IHC Reagents

Primary Antibody

- Rabbit Anti-Rat μ Opioid Receptor/OPRM1 Monoclonal Antibody (R&D Systems, Catalog # MAB8629), 8 μg/mL

Secondary Antibody

- Alexa Fluor® 488-conjugated anti-rabbit secondary antibody, 1:100 dilution

Wash Buffer

- PBS with Tween® 20 (PBST)

Mounting Media

- ProLong™ Gold Antifade Mountant with DAPI

Image Collection

Images were collected with an Olympus Provis fluorescence microscope equipped with a DP71 digital camera.

Tissue Staining Workflow

ISH Protocol

Cut frozen tissue sections

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RNAscope® Target Retrieval
5 minutes in a vegetable steamer

↓

RNAscope® Protease Plus Incubation
20 minutes at room temperature (CRITICAL STEP!)

↓

Probe Hybridization
2 hours in a 40˚C HybEZ oven

↓

Amp 1-FL Hybridization
30 minutes in a 40˚C HybEZ oven

↓

Amp 2-FL Hybridization
15 minutes in a 40˚C HybEZ oven

↓

Amp 3-FL Hybridization
30 minutes in a 40˚C HybEZ oven

↓

Amp 4-FL Hybridization
15 minutes in a 40˚C HybEZ oven

IHC Protocol

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Primary Antibody Incubation
overnight at 4˚C

↓

Wash 2 times in PBST

↓

Secondary Antibody Incubation
1 hour at room temperature

↓

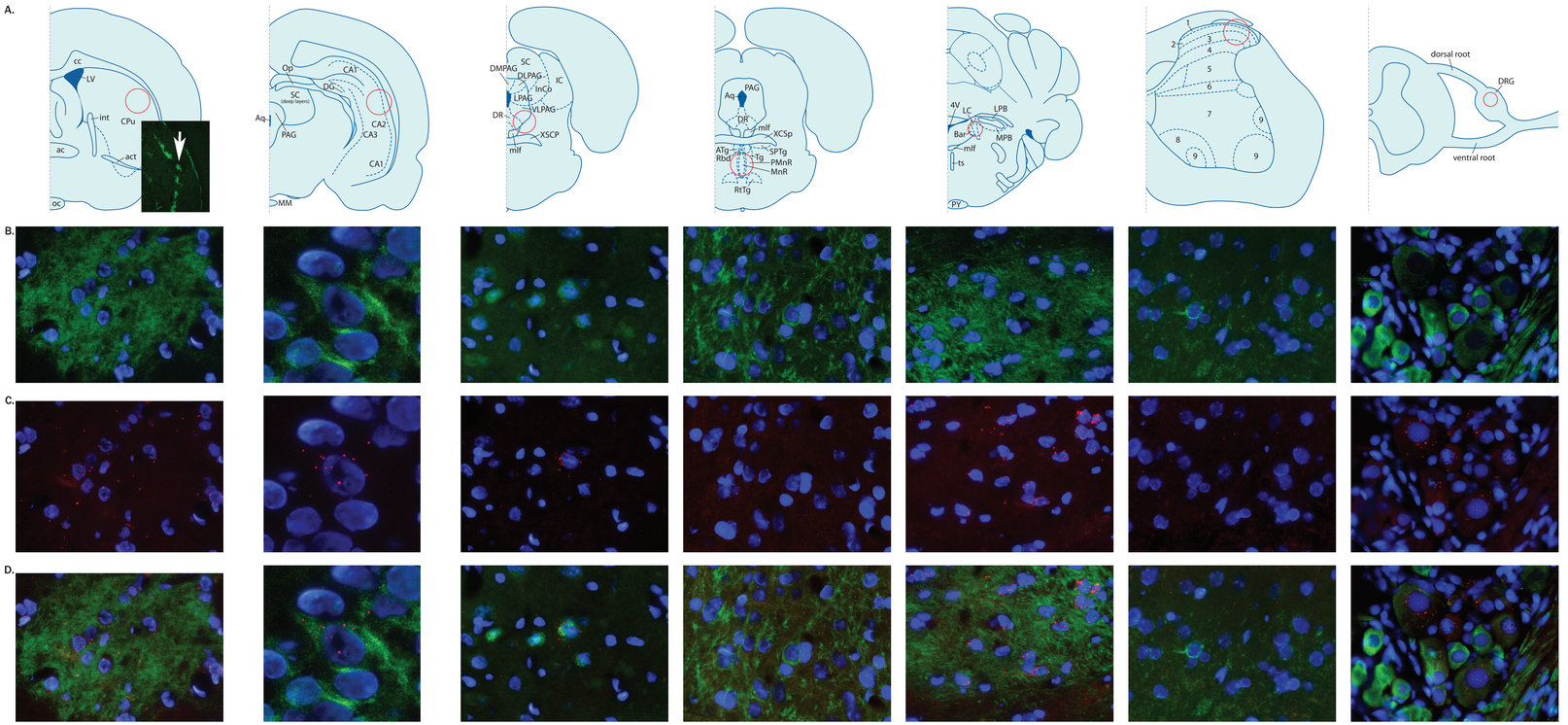
Wash 2 times in PBST

↓

Mount under coverslips

Results

Figure 1. CPu | Figure 2. CA1 | Figure 3. VLPAG | Figure 4. MnR | Figure 5. LC | Figure 6. L3 | Figure 7. DRG



Figures 1–7. Localization of MOR-1A mRNA and OPRM1 protein in select brain and spinal cord regions. **A)** Schematic image illustrating tissue region probed. Red circle outlines the area examined. The arrow on the inset in Figure 1A points to the region that is shown at higher magnification in Figures 1B, 1C and 1D. **B)** IHC detection of OPRM1 protein expression (green). **C)** ISH detection of MOR-1A mRNA expression (red). **D)** Overlap of OPRM1 and MOR-1A labeling. Tissue was counterstained with DAPI (blue).

Brain and Spinal Cord Regions Examined

CA1 CA1 region of hippocampus

CPu caudate putamen

DRG dorsal root ganglia

L3 dorsal horn at the lumbar L3 level of the spinal cord

LC locus coeruleus

MnR median raphe nucleus

VLPAG ventrolateral periaqueductal gray

Results Summary

We observed a low number of double-labeled neurons in the brain cortex (data not shown), CPu, and CA1 region of the hippocampus. More profound double-labeling was observed in the VLPAG and medulla oblongata, which are parts of the descending antinociceptive brainstem circuit. We did not detect any ISH-positive neurons in the MnR. In all the brain and spinal cord regions that were examined, we observed that all ISH-positive neurons were also IHC-positive. The one exception was the DRG. In the DRG, we saw a small number of large-sized ISH-positive neurons that did not display any OPRM1 protein immunoreactivity. Additionally, there was a large number of small-sized neurons in the DRG that showed OPRM1 protein expression but no MOR-1A mRNA expression.

Conclusions

- The large number of ISH- and IHC-positive neurons in the brain and spinal cord regions examined indicate that our rabbit monoclonal antibody is highly specific for rat OPRM1.
- The lack of MOR-1A mRNA in small-sized DRG neurons that stained positive for the OPRM1 protein suggest that these neurons may express other splice variants of the OPRM1 gene.
- The lack of OPRM1 protein expression in large-sized ISH-positive neurons in the DRG suggests that translation of the MOR-1A mRNA in these neurons may be inhibited.
- Using OPRM1 antibodies in combination with probes for different OPRM1 gene splice variants provides a technique for determining the frequency of neurons that express different OPRM1 splice variants.
- The combination of IHC with ISH provides a powerful research technique for the simultaneous detection of a gene's transcript and protein at the single-cell level.

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