8

Nonradioactive In Situ Hybridization in Combination with Tract-Tracing

RUTH L. STORNETTA and
PATRICE G. GUYENET

INTRODUCTION
METHODOLOGICAL CONSIDERATIONS
Riboprobe Design
Production of cDNA Clone
Transformation of cDNA Plasmid into Competent Cells (*E. coli*)
Production of Linear cDNA Template
In Vitro Transcription of cRNA from Linear DNA Template
Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip
Tissue Preparation
In Situ Hybridization
Immunocytochemistry for Revealing Digoxigenin and Other Proteins or Tract-Tracers of Interest
Modifications for Double ISH
Controls
APPLICATIONS
Tract-Tracer Combined with ISH: VGlut1[specific] mRNA-Containing Neurons in Medulla Project to Cerebellum
Tract-Tracer Combined with ISH and Immunocytochemistry: Catecholaminergic Neurons in Rat Medulla Oblongata Containing VGlut2 mRNA Project to Spinal Cord
Tract-Tracer Combined with Double ISH: Neurons Containing Both Preproenkephalin and VGlut2 mRNAs Project to Phrenic Motor Nucleus

RUTH L. STORNETTA AND PATRICE G. GUYENET • Department of Pharmacology, University of Virginia, Charlottesville, VA 22908

Tract-Tracer Combined with ISH and c-Fos: Baroactivated Neurons in Rostral Ventrolateral Medulla Contain PPE mRNA and Project to the Spinal Cord
ISH Combined with Juxtacellular Labeling: Barosensitive, Spinally Projecting Neurons of the Rostral Ventrolateral Medulla Contain PPE mRNA
Viral Tracing Combined with Double ISH: Some Presympathetic Neurons of the Rostral Ventrolateral Medulla Contain Markers of GABA and Glycine
ADVANTAGES AND LIMITATIONS
Advantages Over Radioactive Methods or Antibodies
Disadvantages
APPENDIX: DETAILED PROTOCOLS
In Vitro Transcription of cRNA from Linear DNA Template
Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip
Prehybridization
Hybridization
Rinsing Through Decreasing Salt Concentrations, RNAse A, and High Stringency Wash
Immunocytochemistry for Revealing Digoxigenin and Other Proteins or Tract-Tracers of Interest
Modifications for Double ISH
REFERENCES

Abstract: The use of in situ hybridization (ISH) for the detection of mRNAs in cell bodies has greatly expanded our ability to detect cellular phenotypes in the central nervous system. Riboprobes have been used in the past to identify neuropeptide precursors, distribution of receptors, ion channels, and enzymes. More recently, the discovery of unambiguous markers for the major ionotropic transmitters has made possible the definitive identification of neurons involved in fast transmission. The advantages and disadvantages of different types of probes, including DNA probes, oligonucleotides, and RNA probes for the detection of mRNAs are described. Although in situ hybridization was pioneered with the use of radioactive probes, nonradioactive alternatives are now readily available. The relative merits of nonradioactive probes, specifically for combination with tract-tracing, are discussed. This chapter focuses on in situ hybridization methods based on nonradioactive riboprobes and their use in combination with tract-tracing and immunocytochemistry.

Keywords: c-Fos, clone, digoxigenin, double in situ hybridization, Fluorogold, juxtacellular, plasmid, reverse transcription polymerase chain reaction, riboprobe

I. INTRODUCTION

The use of in situ hybridization (ISH) for the detection of mRNAs in cell bodies has greatly expanded our ability to detect cellular phenotypes in the central nervous system (CNS). The first descriptions of synthetic-labeled

RNA hybridizing to DNA in tissue (Gall and Pardue, 1969; John *et al.*, 1969) enabled the detection of specific DNA sequences not just on a gel or blot but in preserved intact specimens of the native tissue. Some of the first studies to use the ISH technique in brain tissues were performed in the 1980s and focused on the identification of various neuropeptides (Bloch *et al.*, 1986a, b; Hoefler *et al.*, 1986; Lanaud *et al.*, 1989; Pochet *et al.*, 1981; Shivers *et al.*, 1986; Siegel and Young, 1985; Terenghi *et al.*, 1987). Researchers have also been able to localize the mRNA coding for ion channels (Baldwin *et al.*, 1991; Brysch *et al.*, 1991; Hwang *et al.*, 1992; Lenz *et al.*, 1994; McKinnon, 1989; Perney *et al.*, 1992; Rudy *et al.*, 1992; Wang *et al.*, 1994), receptors (Goldman *et al.*, 1986; Malherbe *et al.*, 1990; Rogers *et al.*, 1991; Surmeier *et al.*, 1992; Wada *et al.*, 1988), and enzymes characteristic of certain neurotransmitters (Chesselet *et al.*, 1987; Julien *et al.*, 1987; Mezey, 1989; Seroogy *et al.*, 1989; Wuenschell *et al.*, 1986). More recently, the discovery of unambiguous markers for the major ionotropic transmitters γ-aminobutyric acid (GABA) (Esclapez *et al.*, 1994), glycine (Poyatos *et al.*, 1997), and glutamate (Fremeau *et al.*, 2004) has made possible the definitive identification of neurons involved in fast transmission. RNA detection in neuronal cell bodies is critical for the glutamate vesicular transporters 1 and 2 (VGlut1 and VGlut2) as well as for the glycine transporter (GlyT), since these proteins are present only in terminals and are not found in cell soma. GAD-65 and GAD-67 are also not as readily detected in cell bodies in the brainstem as the mRNA coding for these substances. Thus, the use of ISH has proved to be a critical technique for the identification of the cells involved in fast neurotransmission in the brain (Guyenet *et al.*, 2004).

The cytoplasmic localization of mRNA is an ideal technique when used in combination with retrograde tract-tracing, since the location and phenotypic identification of the projecting neurons is the goal of many tract-tracing studies. With the recent avalanche of sequence information now available, DNA templates may be generated for any known sequence and used for ISH. This technique is much faster and a positive outcome more likely than the generation of specific antibodies for a protein of interest. ISH also has the advantage of a somatic localization, unlike many proteins that are not present in large amounts in the soma, but transported to terminals or assembled into native form in the terminals. Of course, if one is interested in anterograde projections and determining the phenotype of terminal fields, ISH will not be useful.

The early studies describing ISH often relied on DNA probes. These had several difficulties including more labor-intensive cloning techniques. The probe itself was double-stranded and therefore could reanneal after denaturing and reduce the available hybridization sites (Lewis and Baldino, 1990). The engineering of a convenient vector incorporating the bacteriophage promoter next to a multiple cloning site featuring common restriction endonucleases allowed for the creation of a DNA template for the in vitro synthesis of RNA probes (Melton *et al.*, 1984). The RNA probe has several advantages. It is easier to procure the clone for the DNA template. The RNA probe is single-stranded and thus will not hybridize to itself. The

RNA–RNA hybrid formed in the tissue is stronger than a DNA–RNA hybrid and can withstand more stringent rinsing, resulting in lower background. The RNA–RNA hybrid will also resist the action of RNAse, another treatment that will substantially lower background. The use of oligonucleotide probes (Lewis *et al.*, 1985) is a further development in bringing the technique of ISH into laboratories with less familiarity with molecular biological techniques. Synthetic oligonucleotides are widely available, relatively inexpensive, and easy to label with either radioactive or nonradioactive methods. Radioactive-labeled oligonucleotides have been used successfully for many of the same mRNAs as the longer riboprobe counterparts including receptors and channels (Brysch *et al.*, 1991; Pelletier *et al.*, 1988; Wisden *et al.*, 1988). The major drawbacks with oligonucleotide probes are their relative lack of sensitivity for messages expressed at lower levels as well as the possibility for nonspecificity if the detected sequence has many identical regions to another sequence or splice variant.

Although ISH was pioneered with the use of radioactive probes, nonradioactive alternatives are now readily available. The caveat for nonradioactive probes is that they are generally less sensitive due to the lower incorporation of the digoxigenin-labeled nucleotide. However, this is not always the case, and in direct comparisons of some riboprobes, nonradioactive probes were reported to be equally sensitive to their radioactive counterparts (Clavel *et al.*, 1991; Kreft *et al.*, 1996; Mitchell *et al.*, 1993; Park *et al.*, 1991). Low expression levels are not a problem for the neuropeptide precursors, the cytoplasmic enzymes, or the vesicular transporters; however, it is a problem for messages that are expressed at a lower level, e.g., most of the receptors and ion channels. Some of this difficulty may be overcome by making the template longer (i.e., more base pairs), thus offering a greater amount of potential incorporation sites for the labeled nucleotide. On the positive side, the resolution of the nonradioactive method is higher since the signal is expressed directly within the cytoplasm and not as silver grains in an emulsion media overlying the cell. However, the problem of signal-to-noise ratio for low signal level must be realized. Also, if one is interested in quantitative analysis of message levels, the radioactive method is essential. However, for neuroanatomical studies, nonradioactive riboprobes and oligonucleotides can easily be combined with more traditional immunocytochemical methods for the detection of proteins as well as tract-tracing to discover connections of cells with specific phenotypes within the CNS (Johnson *et al.*, 2002; Stornetta *et al.*, 1999, 2002, 2003; Stornetta and Guyenet, 1999). The use of well-designed cDNA clones also provides a reliable source of material, free from the inconsistencies, availability, and specificity issues of polyclonal antibodies.

This chapter focuses on ISH methods based on nonradioactive riboprobes and their use in combination with tract-tracing and immunocytochemistry. For methods for oligonucleotides and/or radiolabeled probes, there are many other excellent references (see Chesselet, 1990; Darby, 2000; Valentino *et al.*, 1987; Wilkinson, 1998; Wisden and Morris, 1994; Young, 1990).

II. METHODOLOGICAL CONSIDERATIONS

A. Riboprobe Design

While it may be easy to obtain clones from colleagues, it is also relatively easy to design and produce a cDNA clone for a particular sequence of interest. This is often faster than waiting for the clone from a busy researcher, who may have problems shipping the clone overseas and has the distinct advantage of not having to sign material transfer agreements or involvement with other obligations. The NCBI Web site (http://www.ncbi.nlm.nih.gov/) with access to many different genetic databases is publicly available. Once the sequence of interest is found, the portion least likely to overlap with other closely related sequences can be determined by using the BLAST search (also found on the NCBI Web site) and noting where similar sequences align. Choose the least similar portion of the sequence for the cDNA template. We have had success with templates up to 3.3 kb in length.

B. Production of cDNA Clone

Design primers for reverse transcription polymerase chain reaction (RT-PCR) of RNA. Primer design is often available online from companies that offer primer synthesis. We have used the PrimerQuest tool from Integrated DNA Technologies (http://www.idtdna.com/SciTools/SciTools.aspx) by Steve Rozen and Helen Skaletsky with code available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html. To create the template, use polyA+ selected RNA (in our case, the RNA is from whole brain or from medulla oblongata). Kits to extract oligo-dT isolated RNA are commercially available from many companies. We currently use a kit from Invitrogen (Carlsbad, CA; FastTrack 2.0 for isolation of mRNA from 0.4–1 g of fresh tissue). Prepared RNA is also commercially available. The next step is the RT-PCR to extract the particular sequence of interest from the sample RNA. We have used the Titan One Tube RT-PCR kit from Roche Applied Science (Indianapolis, IN) according to their directions with very good success. A single band of DNA of the correct length is necessary for use as a good template (Fig. 8.1, Step 1d). Performing a melting curve experiment on the RT-PCR by varying the annealing temperature of the PCR reaction will help in achieving this goal. Once the PCR product is obtained, subclone it into a vector usable for RNA in vitro transcription. We have had very good luck with the pCRII-TOPO vector from Invitrogen following the manufacturer's directions (Fig. 8.1, "RT-PCR," Steps 1a–d).

C. Transformation of cDNA Plasmid into Competent Cells (*E. coli*)

Whether the cDNA plasmid is obtained from outside the laboratory or within the laboratory as detailed in the previous steps, it must be transformed

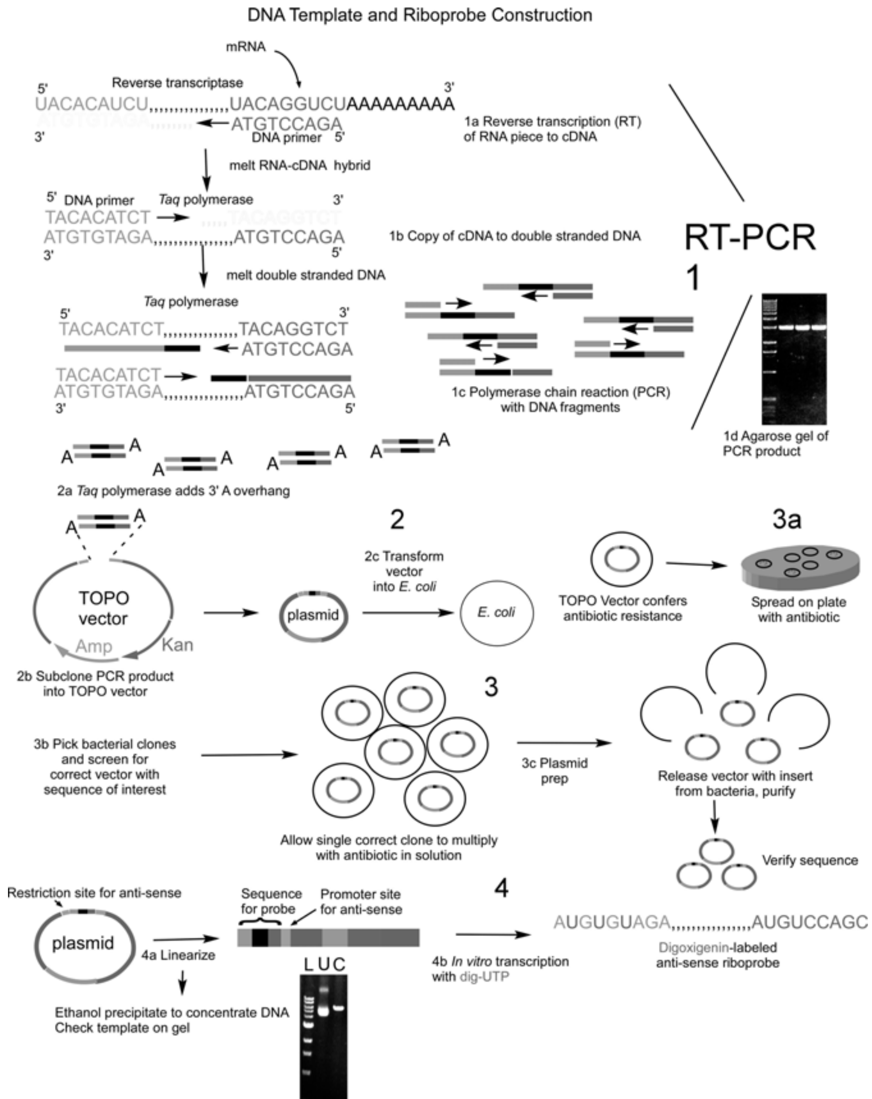

Figure 8.1. Overview of fabrication of DNA template and digoxigenin-labeled riboprobe. Step 1a: Annealing the DNA oligo that is 5′ to 3′ to the mRNA from a sample tissue extract of RNAs and creating a cDNA "copy" of the mRNA sequence of interest catalyzed by the reverse transcriptase enzyme. The RNA–cDNA hybrid melts (becomes single-stranded) by raising the temperature in the thermal cycler (PCR machine). Step 1b: Annealing the DNA oligo that is 3′ to 5′ to the cDNA (instead of the mRNA) and the copying of the cDNA to double-stranded DNA catalyzed by Taq polymerase. Once the copy is made, the cDNA–cDNA hybrid melts by raising the temperature of the PCR machine. Step 1c: The second annealing step is repeated 25–35 times to generate exponential quantities of cDNA. Step 1d: Agarose gel of DNA from PCR reactiona–single band of the correct length is produced. Step 2a: Taq polymerase adds a 3′ A overhang to the cDNA it copies. This property of the cDNA is exploited by the TOPO vector. Step 2b: The PCR product is subcloned into the

into competent cells and prepared in a reasonable quantity for further manipulation. This can be achieved with several commercially available kits. We currently use the One Shot Top 10 F' chemically competent *E. coli* from Invitrogen and the Wizard Plus Midipreps DNA purification system from Promega (Madison, WI) according to the manufacturer's instructions. After larger scale preparation of the DNA, the sequence should be verified before continuing, particularly in reference to the orientation of the clone in the vector. The concentration of the DNA can be determined with a spectrophotometer (Fig. 8.1, Steps 3a–c).

D. Production of Linear cDNA Template

Vectors useful for in vitro RNA transcription have multiple restriction sites on either end of the cloning site of the sequence of interest. Choose a restriction enzyme that cuts only once at the end of the sequence of interest (usually in the multiple cloning site) at the opposite end from the desired promoter site. The enzyme should leave a 5′ overhang or blunt end (Fig. 8.1, Step 2a). (Note: Enzymes that leave a 3′ overhang will result in the production of double-stranded RNA molecules, drastically reducing the yield of the in vitro transcription reaction.) The pCRII-TOPO vector has promoters on either side of the cloning site, thus allowing the production of either sense template or antisense template. We normally set up a large number of restriction enzyme reactions with about 40 μg of DNA total. Our experience is that the restriction enzymes work better in smaller volumes (about 20–30 μl per reaction). It is absolutely critical that the restriction

Figure 8.1. (*Cont.*) TOPO vector (antibiotic resistance areas of the TOPO vector are indicated as "Kan" for kanamycin and "Amp" for ampicillin). The resulting circular piece of DNA (plasmid) is then mixed with chemically competent *E. coli*, incubated on ice for 5–30 min, the reaction heated to 42°C for 30 s, and then returned to the ice. Step 2c: The resulting transformation (the plasmid that is now incorporated into the *E. coli*) is spread on plates (previously prepared with a mix of antibiotic, agar, and appropriate growth medium). Step 3a: After sitting in a 37°C oven overnight, the plates will have small white dots (colonies of bacterial clones). Individual colonies are picked with a sterile toothpick or wire loop and placed into an aliquot of liquid growth media containing appropriate antibiotic. Step 3b: Screen a sample of the colonies to determine whether the colony contains the correct plasmid before growing the colony in large quantities. Step 3c: The "plasmid prep" is the procedure for growing large quantities of the correct bacterial colonies, and then releasing the plasmids from the bacteria and purifying the plasmid DNA. After this process, the plasmid sequence must be verified. Step 4a: The correct purified plasmid DNA is linearized with an appropriate endonuclease (restriction enzyme). This linearized DNA serves as the template and is concentrated with ethanol and checked on an agarose gel (L = ladder, U = uncut DNA plasmid, C = linear DNA template). Step 4b: The linear DNA is then transcribed into digoxigenin-labeled cRNA (riboprobe) in vitro using the appropriate RNA polymerase in a solution containing digoxigenin-labeled UTP.

enzyme cuts the DNA to completion. Any traces of circular plasmid DNA remaining will carry over to the in vitro transcription reaction, resulting in long stretches of noncoding plasmid sequence transcription and incorporation of much of the labeled nucleotide into this "garbage" sequence. Check the completion of the reaction by gel electrophoresis of 1 μl from each enzyme reaction as well as 1 μl of uncut ("supercoiled") plasmid DNA (Fig. 8.1, Step 4a). The uncut DNA will run at different lengths than the cut DNA. There should be only one clear band per restriction enzyme reaction, with no bands appearing like the uncut DNA. Combine all successful restriction reactions into one tube and perform a phenol–chloroform extraction to eliminate the enzyme and any other impurities from the now-linearized template. Concentrate the DNA template by ethanol precipitation. Measure the concentration by spectrophotometer (Fig. 8.1, Step 4a).

E. In Vitro Transcription of cRNA from Linear DNA Template

Assemble the components of the reaction mixture (with the exception of the enzyme) at room temperature and in the stated order to prevent the precipitation of DNA template by spermidine in the reaction buffer. Be aware of keeping everything as clean and RNAse-free as possible—use gloves and sterile tips and do the reaction assembly on a clean surface. Mix ingredients thoroughly in a sterile plastic Eppendorf tube by pipetting up and down after each addition (see Appendix section "In Vitro Transcription of cRNA from Linear DNA Template" for detailed recipes).

F. Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip

This step is necessary to determine whether the riboprobe has been labeled with digoxigenin and also to determine the relative amount of digoxigenin that has been incorporated (see Appendix section "Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip" for further details).

G. Tissue Preparation

Anesthetize rats with pentobarbital and perfuse transcardially with 100 ml phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. Extract brains and postfix in the same fixative for up to 3 days at 4°C (postfixation does not appear to affect ISH; however, take care for postfixation time for any additional antibodies used). Section brains at 30 μm on a vibrating microtome at room temperature in 0.1 M phosphate buffer. (Brains may also be sunk in 25% RNAse-free sucrose and cut frozen.)

Collect sections in RNAse-free cryoprotectant (50 mM sterile phosphate buffer, 30% ethylene glycol (Sigma-Aldrich, St. Louis, MO), 20% glycerol (RNAse-free; Sigma-Aldrich)) in sterile 24-well tissue culture plates. Sections may be kept in this solution at −20°C for up to 1 year.

H. In Situ Hybridization

1. Prehybridization

This step allows the tissues to adapt to the conditions of the hybridization buffer as well as serving as an important blocking step to prevent or at least decrease nonspecific hybrid formation. It is extremely important to make the prehybridization mixture with sterile, RNAse-free solutions, and with sterile dishes, pipette tips, etc. This is the time to be paranoid about RNAse! (see Appendix section "Prehybridization" for details).

2. Hybridization

In this step, the riboprobe is added directly to the "prehybridization" solution, and the conditions are optimized for hybrid formation. See Fig. 8.2 for a summary and Appendix section "Hybridization" for further details.

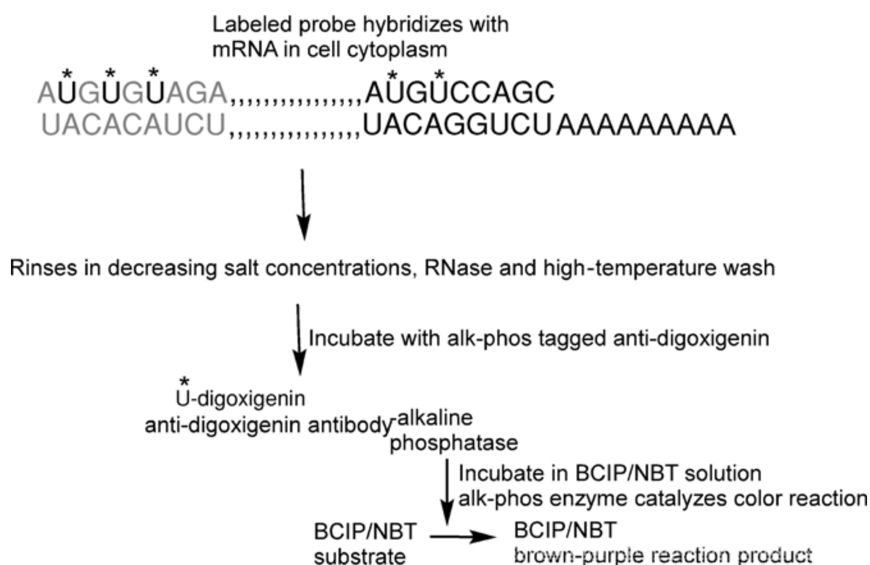

Figure 8.2. Overview of visualization of digoxigenin-labeled riboprobe.

3. Rinsing

The rinsing of the tissue after the hybrids are formed is necessary to decrease the background (nonspecific hybrids) as well as to destroy any riboprobe that is not hybridized. The basic idea is to take the tissue through solutions of decreasing salt concentration as the RNA–RNA hybrid is sensitive to salt concentration. Weaker bonds (i.e., nonspecific hybrids) will not survive the lower salt concentrations. One of the rinsing steps involves treatment with RNAse A, an enzyme that will destroy single-stranded RNA (e.g., nonhybridized riboprobe). The final step is a high-temperature rinse in the lowest concentration of salt solution, a condition in which only the strongest, most specific hybrids will survive. For a summary, see Fig. 8.2, and for details, see Appendix section "Rinsing Through Decreasing Salt Concentrations, RNAse A, and High Stringency Wash."

I. Immunocytochemistry for Revealing Digoxigenin and Other Proteins or Tract-Tracers of Interest

After the hybrids are formed and the tissue is rinsed, the hybrids are stable and proteins of interest may be revealed by using standard immunocytochemical protocols. The digoxigenin label as well as the Fluorogold (FG) tracer is revealed in this manner. See Fig. 8.2 for a summary and see Appendix section "Immunocytochemistry for Revealing Digoxigenin and Other Proteins or Tract-Tracers of Interest" for details.

J. Modifications for Double ISH

A second nonradioactive cRNA with a different sequence of interest can be transcribed, substituting FITC-12-UTP (Roche) for the digoxigenin-11-UTP (see Appendix protocol "In Vitro Transcription of cRNA from Linear DNA Template" for further details). To test the FITC-labeled riboprobe on the dot blot, substitute sheep anti-FITC peroxidase-tagged antibody (1:2000; Roche) for the sheep alkaline phosphatase (AP) tagged anti-digoxigenin antibody. For further details, see Appendix protocol G.1; for further details about visualizing the FITC probe for ISH, see Appendix protocol G.2.

K. Controls

1. Hybridization with the Sense Strand of cRNA

After in vitro transcription of the sense strand of cRNA, compare the incorporation of digoxigenin of both sense and antisense cRNAs by relative

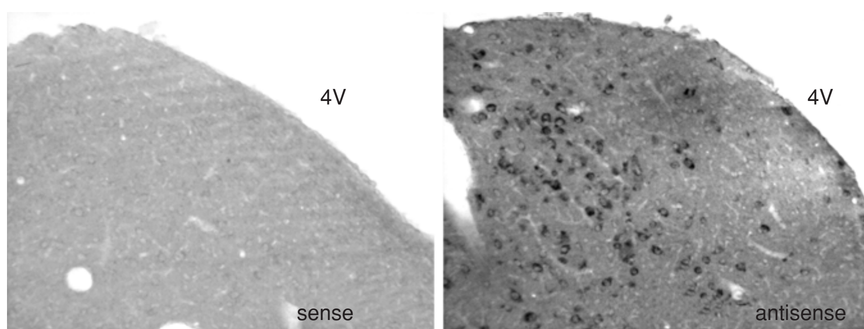

Figure 8.3. Sense/antisense control with cRNA for vesicular glutamate transporter-2 in medial vestibular nucleus of rat medulla oblongata.

appearance on the dot blot. Use a concentration about 2–3×, the equivalent amount of antisense cRNA, and do "side by side" ISH on tissue from the same brains with sense and antisense riboprobes. Allow the colorization procedure to progress for the same amount of time. There should be very strong signal in appropriate places for the antisense riboprobe-hybridized tissues and no signal in the sense-hybridized tissues (see Fig. 8.3). This is the standard control for specific hybridization signal.

2. Hybridization Signal in Expected Areas but No Signal Where No mRNA Should Be Found

We feel that the neuroanatomical consistency of the signal in areas that are known to express the mRNA versus no signal in areas where no mRNA is present is the best control (see Fig. 8.4). This is difficult in cases where the mRNA is ubiquitously or very broadly expressed. In these cases, one must rely on the sense/antisense control data. The internal consistency of the mRNA being expressed in certain types of neurons is also helpful (e.g., in the case of the VGlut2, we never saw the mRNA expressed in GABAergic neurons). One of the standards we have used for many mRNAs of interest is the lack of expression of mRNA in motor neurons (the larger motor neurons are notorious for expressing artifactual background in immunohistological procedures).

III. APPLICATIONS

A. Tract-Tracer Combined with ISH: VGlut1[specific] mRNA-Containing Neurons in Medulla Project to Cerebellum

After noting the striking distribution of VGlut1 mRNA in medulla oblongata in precerebellar nuclei and the fact that VGlut1 protein is expressed in

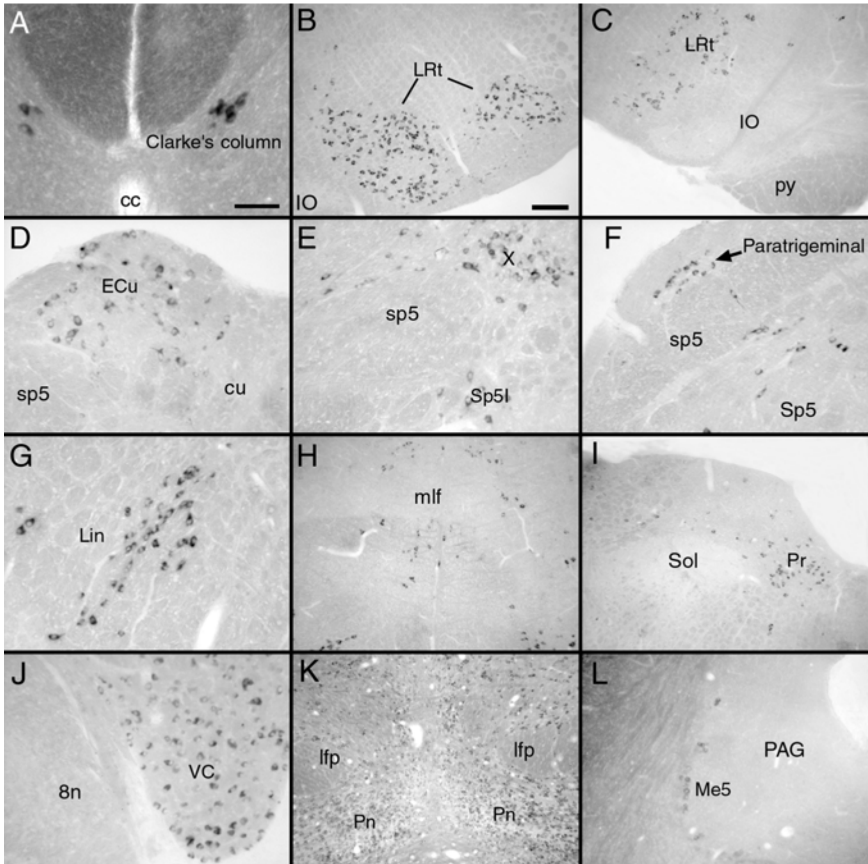

Figure 8.4. Discrete distribution of VGlut1 mRNA in rat brain and spinal cord. (A) Clarke's column of dorsal horn of spinal cord. (B) Lateral reticular nucleus (IO, inferior olive). (C) Lateral reticular nucleus [note the lack of label in the IO and the pyramidal tract (py)] (D) External cuneate nucleus (ECu) (sp5, spinal trigeminal tract; cu, cuneate fasciculus). (E) Spinal trigeminal tract and nucleus X. (F) Ectotrigeminal nucleus (E5) and paratrigeminal nucleus. (G) Linear nucleus (Lin). (H) Medial brainstem at Bregma level −12.80 mm (mlf, medial longitudinal fasciculus). (I) Prepositus nucleus (Pr) (Sol, nucleus of the solitary tract). (J) Ventral cochlear nucleus (VC) (8n, eighth nerve). (K) Pontine nucleus (Pn) (lfp, longitudinal fasciculus pons). (L) Mesencephalic trigeminal nucleus (Me5) (periaqueductal gray, PAG). Scale bar: 100 μm for A, D, E, G, J, and L. Scale bar:200 μm for B, C, F, H, I, and K.

mossy fibers in cerebellum (Bellocchio *et al.*, 1998; Hisano *et al.*, 2002), we tested the hypothesis that most cerebellar-projecting neurons in rat brainstem contain VGlut1 mRNA. Four 100-nl pressure injections of the retrograde marker FG (2% in sterile saline; Fluorochrome Inc., Englewood, CO; Schmued and Fallon, 1986) were placed 1–2 mm deep in various

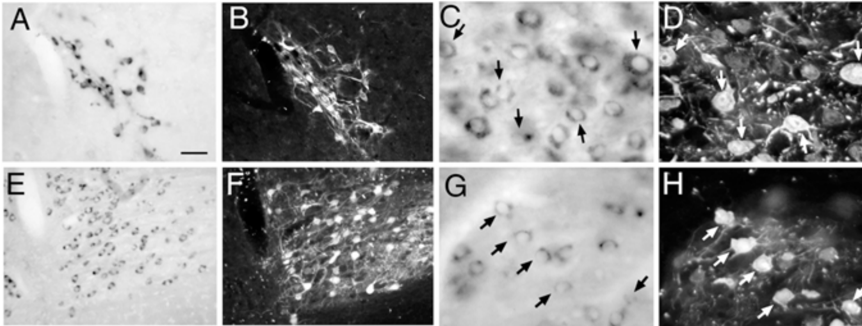

Figure 8.5. Examples of colocalization of VGlut1 mRNA and Fluorogold (FG) (from cerebellum). (A) VGlut1 mRNA in linear nucleus (brightfield). (B) FG immunoreactivity (Cy-3 revealed by fluorescence; same field as in A). Note that most of the cells are double labeled. (C) VGlut1 mRNA in pontine nucleus (brightfield). (D) FG immunoreactivity (Cy-3 revealed by fluorescence; same field as in C). Arrows point to a few of the many double-labeled cells. (E) and (G) VGlut2 mRNA in inferior olive (brightfield). (F) and (H) FG immunoreactivity (Cy-3 revealed by fluorescence; same field as in E). Arrows point to some of the double-labeled cells. Scale bar: 50 μm for A, B, E, and F. Scale bar: 20 μm for C, D, G, and H.

locations of the left cerebellar cortex. The brainstem was processed for ISH for VGlut1 mRNA in combination with FG immunocytochemistry. [Immunocytochemical detection of FG was accomplished by incubating the tissue with a rabbit anti-FG antibody (1:10,000; Chemicon, Temeluca, CA), followed by a biotinylated anti-rabbit IgG (1:200; Vector, Burlingame, CA) and visualized with streptavidin Cy3 (1:1000; Molecular Probes, Eugene, OR).] We found that the vast majority of FG-labeled neurons in pons and medulla (with the exception of the inferior olive) contained VGlut1 mRNA (Fig. 8.5).

B. Tract-Tracer Combined with ISH and Immunocytochemistry: Catecholaminergic Neurons in Rat Medulla Oblongata Containing VGlut2 mRNA Project to Spinal Cord

Fluorogold (2–3% in sterile saline) was pressure injected into the vicinity of the intermediolateral cell column (1 mm below the entry point of the dorsal roots) at the first and third thoracic segments bilaterally (50-nl injections, four injections per rat). The brainstem was processed for ISH with VGlut2 riboprobe in combination with antibodies for tyrosine hydroxylase (mouse monoclonal; Sigma) and FG (described above). We found many neurons immunoreactive for tyrosine hydroxylase that also contained VGlut2 mRNA and FG. Some of these neurons are illustrated in Fig. 8.6. This was the first study to demonstrate that brainstem presympathetic catecholaminergic neurons are glutamatergic.

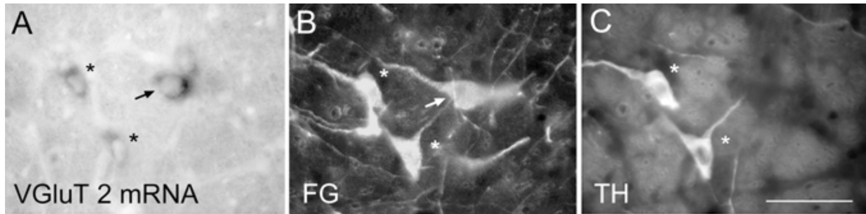

Figure 8.6. Rostral ventrolateral medulla (RVLM) catecholaminergic and noncatecholaminergic neurons with projection to the thoracic spinal cord contain VGlut2 mRNA. (A–C) RVLM neurons in the same field photographed under brightfield in A show VGlut2 mRNA AP reaction product and under fluorescent light in B and C show FG immunoreactivity (ir) revealed with Cy-3 (B) and tyrosine hydroxylase (TH) ir revealed with Alexa 488 (C). Asterisks indicate two TH-ir bulbospinal cells that contain VGlut2 mRNA. The arrow points to a strongly VGlut2-positive bulbospinal cell that is devoid of TH-ir. Scale bar: 50 μm. (From Stornetta *et al.* 2002.)

C. Tract-Tracer Combined with Double ISH: Neurons Containing Both Preproenkephalin and VGlut2 mRNAs Project to Phrenic Motor Nucleus

FluoroGold was injected iontophoretically into the left ventral horn of spinal segment C4 after recording respiratory activity at the injection site. The rat medulla oblongata was processed for ISH with a VGlut2 digoxigenin-tagged riboprobe and a preproenkephalin (PPE) FITC-tagged riboprobe in combination with antibodies for FG (described above). Many bulbospinal neurons of the rostral ventral respiratory group in the caudal medulla oblongata contained both VGlut2 and PPE mRNAs. Examples of these neurons as well as the injection site are shown in Fig. 8.7. This study demonstrated that glutamatergic neurons controlling respiratory output could also use enkephalin as a neurotransmitter.

D. Tract-Tracer Combined with ISH and c-Fos: Baroactivated Neurons in Rostral Ventrolateral Medulla Contain PPE mRNA and Project to the Spinal Cord

FluoroGold was pressure injected into the first and third segments of thoracic spinal cord. Rats were cannulated for chronic blood pressure recording and intravenous injections. Injections of hydralazine into conscious, freely moving rats lowered blood pressure, and after 2 h animals were anesthetized and perfused. The brainstem was processed for ISH for PPE mRNA (detected by a digoxigenin-labeled riboprobe) in combination with antibodies for FG (visualized with Alexa 488) and c-Fos (goat polyclonal, Santa Cruz, visualized with streptavidin Cy3). Many bulbospinal neurons were c-Fos positive (baroactivated) and contained PPE mRNA. Examples of these neurons are shown in Fig. 8.8. This study demonstrates that c-Fos, a

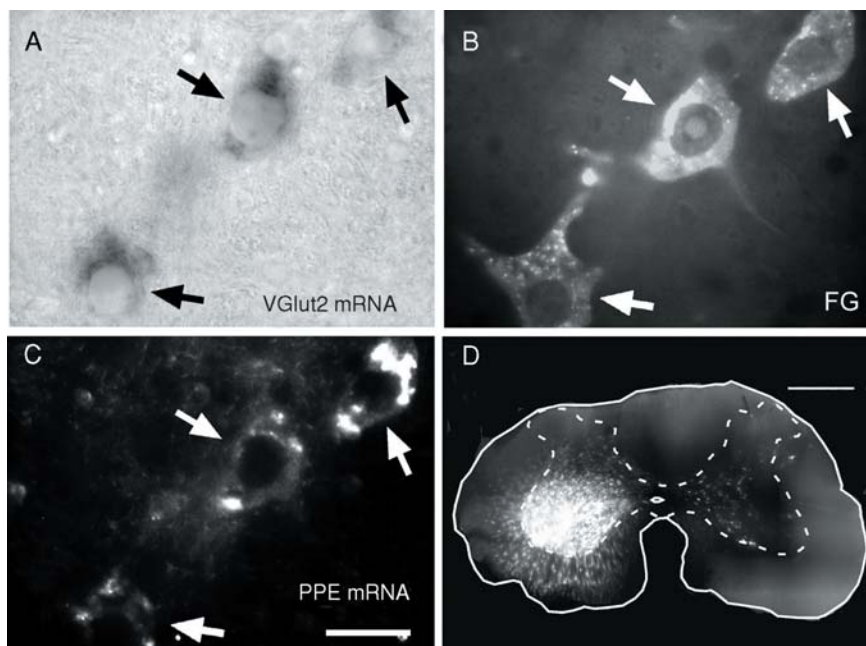

Figure 8.7. Colocalization of VGlut2 mRNA and PPE mRNA in rostral ventral respiratory group (rVRG) bulbospinal neurons. Cluster of rVRG neurons (arrows) containing (A) VGlut2 mRNA (BCIP/NBT reaction product; brightfield), (B) immunoreactivity to Fluorogold (Alexa 488, epifluorescence), and (C) PPE mRNA (Cy3; epifluorescence). Scale bar: 20 μm. (D) Fluorogold injection site into fourth cervical spinal cord segment. Composite photomicrograph of center of iontophoretic deposit. Scale bar: 500 μm. (From Stornetta *et al.*, 2003.)

useful marker of cell activation, can be colocalized with mRNAs of interest as well as with tract-tracers. In this particular example, this was the first demonstration of baroactivated presympathetic enkephalinergic neurons in brainstem.

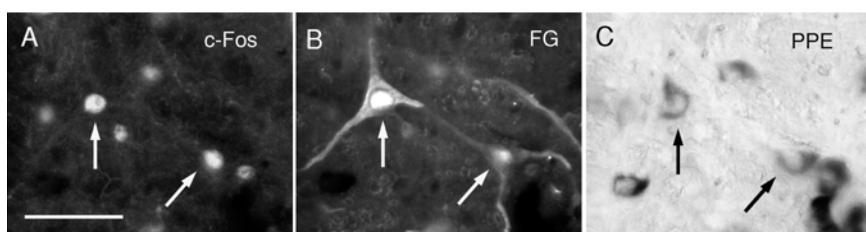

Figure 8.8. Preproenkephalin (PPE), c-Fos immunoreactive (ir) neurons in RVLM project to spinal cord. Cluster of RVLM neurons (arrows) containing (A) Fos-ir (Cy3; epifluorescence), (B) Fluorogold (FG)-ir (Alexa 488, epifluorescence), and (C) PPE mRNA (digoxigenin-labeled probe, brightfield). Scale bar: 50 μm. (From Stornetta *et al.*, 2001.)

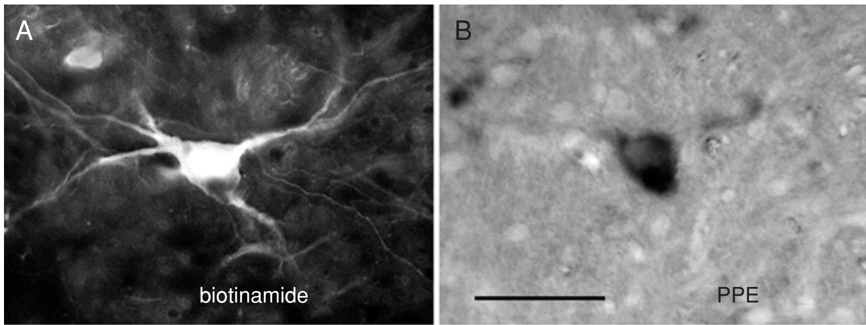

Figure 8.9. Blood pressure–sensitive neuron in RVLM antidromically activated from thoracic spinal cord contains PPE mRNA. (A) Neuron in RVLM recorded in vivo and juxtacellularly labeled with biotinamide (Alexa 488, epifluorescence). (B) Same neuron showing PPE mRNA (digoxigenin-labeled probe, brightfield). Scale bar: 50 μm. (From Stornetta *et al.*, 2001.)

E. ISH Combined with Juxtacellular Labeling: Barosensitive, Spinally Projecting Neurons of the Rostral Ventrolateral Medulla Contain PPE mRNA

Barosensitive vasomotor presympathetic cells were recorded in rostral ventrolateral medulla in vivo in halothane-anesthetized rats. Cells were labeled using the juxtacellular method (described in the chapter by Duque and Zaborszky, this volume) After transcardial perfusion, the brainstem was sectioned and processed for ISH and PPE mRNA (detected by a digoxigenin-labeled riboprobe) in combination with streptavidin Alexa 488 (Molecular Probes) for the detection of the biotinamide-labeled cell. An example of one of these PPE mRNA positive, spinally projecting (detected by antidromic activation from the spinal cord) barosensitive neurons is shown in Fig. 8.9. The ability to find the mRNA of physiologically identified neurons has allowed a major breakthrough in our ability to perform functional neuroanatomy. In this particular example, this was the first demonstration of enkephalinergic presympathetic neurons with firing patterns inversely correlated with blood pressure.

F. Viral Tracing Combined with Double ISH: Some Presympathetic Neurons of the Rostral Ventrolateral Medulla Contain Markers of GABA and Glycine

Rats were anesthetized and the adrenal gland was exposed and injected with pseudorabies virus (PRV; provided by L. Enquist, Princeton University, NJ). This technique is described in further detail in the chapter by Geerling *et al.* (this volume). After 3 days, animals were anesthetized and perfused transcardially. The rat medulla oblongata was processed for ISH with a GAD-67 digoxigenin-tagged riboprobe and a GlyT2 FITC-tagged riboprobe in

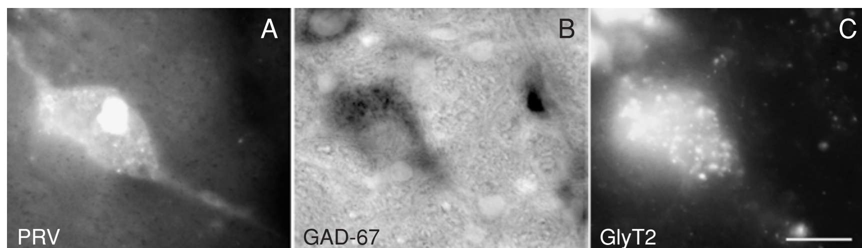

Figure 8.10. Presympathetic neuron in gigantocellular reticular nucleus ventral containing (A) pseudorabies virus (PRV)-ir (Alexa 488, epifluorescence), (B) GAD-67 mRNA (digoxigenin-labeled probe, brightfield), and (C) glycine transporter-2 (GlyT2) mRNA (FITC-labeled probe, Cy3, epifluorescence). Scale bar: 50 μm. (From Stornetta *et al.*, 2004.)

combination with antibodies for PRV (rabbit polyclonal, Enquist). An example of a neuron infected with PRV (resulting from injection in the adrenal gland) in an area of the gigantocellular nucleus in medulla that also contains GAD-67 and GlyT2 mRNAs is seen in Fig. 8.10. This demonstrates that some presympathetic neurons have the capacity for inhibitory control of sympathetic outflow. A major caveat with detection of mRNAs in cells infected with viruses is that viruses corrupt the RNA processing machinery and the native mRNAs are replaced by viral RNAs; thus, allowing only detection of native mRNA at very early time points in the cell's infection with virus (this issue is discussed in more detail in the chapter of Geerling *et al.*, this volume).

IV. ADVANTAGES AND LIMITATIONS

A. Advantages Over Radioactive Methods or Antibodies

1. Detection of nonradioactive riboprobes can be combined with the detection of common retrograde tract-tracers as well as many commercially available antibodies. Nonradioactive probes are particularly well suited for combination with multiple fluorescent tags.
2. Nonradioactive probes can be combined with other techniques such as pseudorabies viral tracing (Stornetta *et al.*, 2004) and juxtacellular labeling (Schreihofer *et al.*, 1999; Stornetta *et al.*, 1999, 2002, 2003).
3. Nonradioactive ISH reveals the transcripts in cell bodies making possible detection of substances normally transported to or solely present in terminals. This is essential for the identification of a particular cell phenotype in combination with retrograde tracers.
4. Nonradioactive ISH does not require special licensing/handling for radioactive materials.
5. Nonradioactive ISH is much faster than radioactive ISH and relative signal-to-noise ratio is easier to control.
6. Nonradioactive riboprobes can be produced in large amounts and kept frozen for at least 1 year before use.

7. Riboprobes can be generated for any known sequence fairly quickly and easily (within 1–2 weeks) compared to several months for antibody production.
8. Riboprobe specificity can be tested with sense controls as well as tissue specificity and can also be subjected to Northern blot analysis.

B. Disadvantages

1. Fluorescent retrograde tracers such as FG fade when exposed to ISH conditions and must be amplified with antibodies for best visualization after ISH.
2. Incorporation of nonradioactive label is less than for radioactive riboprobes and thus detection of low-level messages is more difficult and sometimes impossible with nonradioactive ISH.
3. Attention must be paid to sterile conditions as RNAse can wreak havoc with the procedure.
4. Extra equipment and reagents for molecular biology procedures, including centrifuges, gel electrophoretic apparatus, water baths, and enzymes, must be acquired/borrowed .
5. The protocol requires a few extra days as well as many extra steps over standard immunocytochemical methods.
6. The best available antibody to digoxigenin is AP-tagged and the reaction product of nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) cannot be dehydrated or covered with “permanent” mounting media. The lack of dehydration results in the tissue looking a bit less clear than with peroxidase reaction products that can be dehydrated. This can be overcome by using a riboprobe transcribed with FITC-12-UTP (Roche) and visualized with a sheep anti-FITC antibody tagged with peroxidase and reacted with a traditional peroxidase substrate.

APPENDIX: DETAILED PROTOCOLS

A. In Vitro Transcription of cRNA from Linear DNA Template

A.1. In vitro transcription reaction mixture

Sterile H_2O (q.s. to 90 μl total volume)
1.5 μg DNA template (volume will depend on concentration)
9 μl 100 mM DTT (provided with enzyme)
18 μl ribonucleotides—UTP (ATP, CTP, GTP mix equal parts of 10 mM stock; Promega)
10.8 μl 2 mM UTP (we have tried varying the concentration of the unlabeled UTP—this concentration gives us the best results. Note that some unlabeled UTP is necessary for the reaction to work)

2.1 μl digoxigenin-11-UTP (Roche)
18 μl 5× transcription buffer
3 μl RNA polymerase (T3, T7, or SP6; Promega)

A.2. Incubate reaction in a 500 μl Eppendorf tube in a water bath for 2 h. For T3 or T7, incubate at 37°C. For SP6, incubate at 40°C.

A.3. Add 1.5 μl RQ1 DNase (Promega) and incubate for another 20 min at 37°C. (This will destroy the DNA template.)

A.4. Purify the probe with ProbeQuant G-50 micro columns (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions or by phenol–chloroform extraction and ethanol precipitation (we use the micro columns).

B. Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip

B.1. Use a thin Nytran strip (Nytran Supercharge, SPC, pore size of 0.45 μm; Schleicher and Schuell, Keene, NH) razor cut (~0.5 cm ×6 cm) to fit in a 5-ml plastic test tube.

B.2. Wet the strip by immersion in 100% ethanol for 3 min.

B.3. Rinse in 2X saline-sodium citrate (SSC) (20×SSC stock: 3.0 M NaCl and 0.3 M sodium citrate in water, pH 7.0) for 3 min and air-dry on a piece of filter paper for about 10 min.

B.4. Spot 1-μl aliquots of dilutions of control digoxigenin-labeled RNA (Roche) along with 1-μl aliquots of dilutions of the probe to be tested on the strip (we use 1/100, 1/200, and 1/400).

B.5. Cross-link the RNA to the Nytran membrane with UV light [we use the Stratagene (La Jolla, CA) UV Stratalinker 1800 set on auto cross-link.]

B.6. Immerse the strip in 2–3 ml 2×SSC in the test tube for 3-min shaking.

B.7. Immerse in 2–3 ml phosphate buffer-bovine serum albumin-triton (PBT) [0.1% bovine serum albumin (BSA)/0.2% Triton X-100 in PBS] and incubate for 15 min on shaker.

B.8. Incubate in anti-digoxigenin tagged with AP Fab fragments from sheep (1:1000; Roche) in PBT for 30 min on shaker.

B.9. Rinse 3 × 5 min in PBS.

B.10. Immerse in NMT (0.1 M NaCl/50 mM $MgCl_2$ in 0.1 M Tris, pH 9.5) for 5-min shaking.

B.11. Immerse in solution of NBT (4.5 μl/ml of NMT) and BCIP (3.5 μl/ml of NMT; NBT and BCIP from Roche). Spots should appear momentarily.

B.12. Stop the reaction by rinsing in TE 8.5 (Tris-EDTA). Use the color intensity relative to the control RNA to determine approximate concentration of probe. We find that, generally, the in vitro

translated riboprobe should be about equal to the control RNA for best signal.

C. Prehybridization

C.1. Rinse sections in sterile PBS in sterile petri dishes. We use glass rods fashioned by flaming glass pipettes to seal the ends and make a hook for transferring sections between solutions. Rinse the glass hooks with RNaseZap (Ambion Inc., Austin, TX) prior to use.

C.2. Transfer sections to prehybridization mixture (300 μl per well in a sterile 24-well tissue culture dish). Four to eight sections will fit per well, depending on the size of the tissue. Incubate sections in this solution for 30–60 min shaking at room temperature and then at 37°C for 1 h.

C.3. Prehybridization mixture (in sterile H_2O)

0.60 M NaCl
0.10 M Tris-Cl (7.5)
0.01 M EDTA
0.05% sodium pyrophosphate
5% (w/v) dextran sulfate (must vortex and heat to 37°C to dissolve)
0.50 mg/ml yeast total RNA (Sigma R-7125)
0.05 mg/ml yeast tRNA (Roche 109495)
1×Denhardt's BSA [50X Denhardt's solution: 5 g Ficoll-70 (Sigma), 5 g polyvinylpyrrolidone, 5 g BSA (Fraction V; Sigma) q.s. with H_2O to 500 ml; may be kept in frozen aliquots]
50% deionized formamide (Sigma)
0.05 mg/ml poly A (Sigma P-9403)
10 μm of the four nucleoside triphosphates
10 mM dithiothreitol (DTT; Promega)

Make up prehybridization solution in larger batches and freeze in appropriate-size aliquots. Add 0.5 mg/ml herring sperm (Sigma D-6898-sodium salt of ribonucleic acids from herring testes) that has been boiled for 10 min to denature and quenched on ice just prior to use.

D. Hybridization

D.1. Either add riboprobe directly to wells or transfer the sections into a new solution of prehybridization mixture to which the riboprobe has been added. We found concentrations of 1–3 μl of riboprobe per well (resulting in 50–100 pg/μl) to be most effective for a good signal-to-noise ratio.

D.2. Incubate the sections with riboprobe at room temperature on the shaker for 15 min.

D.3. Incubate at 55–60°C overnight (shaking not necessary). The tissue culture dish cover comes in handy for this—we have never had to worry about any extra precautions to stop evaporation.

E. Rinsing Through Decreasing Salt Concentrations, RNAse A, and High Stringency Wash

E.1. Transfer the sections to a mesh-well bottom dish (Nason Fabrications, Fort Bragg, CA) filled with 4 × SSC/10 mM sodium thiosulfate (NaTS).

E.2. Rinse 2 × 20 min @ 37°C in this solution.

E.3. Using gloves and a work pad, change solution to 20 μg/ml RNAse A (Sigma) in RNAse buffer [0.5 M NaCl, 10 mM Tris (pH 8.0) 1 mM EDTA]

E.4. Incubate @ 37°C for 30 min.

E.5. Change solution to RNAse buffer and incubate @ 37°C for 20 min.

E.6. Transfer to solution of 2 × SSC/10 mM NaTS @ 37°C for 20 min.

E.7. Transfer to a solution of 0.5 × SSC @ 37°C for 20 min.

E.8. Do a final "high-stringency" rinse in 0.1 × SSC at 50–55°C for 30–60 min. If background is a problem, this high-temperature rinse may be increased in temperature up to 60°C.

F. Immunocytochemistry for Revealing Digoxigenin and Other Proteins or Tract-Tracers of Interest

All rinses and incubations done at room temperature on shaker unless noted otherwise.

F.1. Rinse sections 3 × 5 min in TBS [0.1 M Tris (pH 7.4)/0.15 M NaCl]

F.2. Incubate 30 min in 10% normal horse serum/0.1% Triton X-100 in TBS.

F.3. Incubate in sheep anti-digoxigenin tagged with AP (Roche) at 1:1000 in 10% normal horse serum/0.1% Triton X-100 in TBS first for 1 h at room temperature on the shaker and then overnight at 4°C shaking. Centrifuge the digoxigenin antibody prior to use, and use only the supernatant portion. Note: In our hands, the only antibody against digoxigenin that works well is the sheep AP-tagged antibody from Roche.

F.4. Add other antibodies of interest to this same mixture. Fluorescent tract-tracers such as FG (Fluorochrome Inc., Denver, CO) should be amplified by using an antibody against FG (e.g., rabbit anti-FG; Chemicon), since the ISH procedure causes fading of these markers.

F.5. Rinse sections 2 × 10 min in TBS.

F.6. Add direct-tagged fluorescent secondary antibody appropriate to FG or other antibodies at this time and incubate for 45–60 min.

F.7. Rinse 2 × 10 min in TBS.

F.8. Rinse 10 min in NMT (0.1 M NaCl/50 mM $MgCl_2$ in 0.1 M Tris, pH 9.5).

F.9. Transfer sections to a solution of NBT (4.5 μl/ml of NMT) and BCIP (3.5 μl/ml of NMT; 300 μl to 1 ml per well in a sterile 24-well tissue culture dish). Filter the NBT/BCIP solution before use (we use syringe microfilters of 0.22-μm pore size). It is important that the NBT/BCIP solution be in

clean plastic or glassware. Any dust or dirt can cause the reaction to seed, and precipitate will form. Allow this reaction to proceed at room temperature protected from light on the shaker. Check the progress of the reaction after 30 min and then every 15 min until the dark reaction product is seen in cell bodies in appropriate areas and not in areas where the mRNA should not be present. This is critical to the success of the experiment. We use a dissecting microscope to inspect the tissue, while the reaction is progressing to ensure a good signal-to-noise ratio. The reaction should be stopped immediately if any background begins to appear.

F.10. Once there is dark reaction product and very low background (this may take between 45 min and 4 h, depending on the probe and the amount of mRNA expression), stop the reaction by transferring the sections back into the mesh-well dish in a solution of TE, pH 8.5.

F.11. Rinse 3 × 10 min in TE, pH 8.5.

F.12. Rinse 3 × 5 min in TBS and 1 × 5 min in 0.1 M phosphate buffer.

F.13. Mount from 0.1 M phosphate buffer. Air-dry and cover with Vectashield (Vector, Burlingame, CA) or another aqueous-based mounting media. Vectashield is good for protecting any other fluorophores in the reaction (e.g., fluorescent-tagged secondaries) from fading. Seal edges of coverslip with nail polish. Do not dehydrate the sections in alcohols or xylenes. Note: The NBT/BCIP reaction product is soluble in alcohols and organic solvents and is also light sensitive. The color will change slightly as the slides are exposed to light.

G. Modifications for Double ISH

Unfortunately, a commercially available FITC-tagged control RNA is not available to aid in determining exact concentrations of FITC-labeled cRNA from the dot blot test.

G.1. *Dot Blot Nytran Strip Test for FITC Probe.* Follow section "Test for Incorporation of Nonradioactive Label Using a Dot Blot on a Nytran Strip" for Steps B.1–B.7. Use sheep anti-FITC peroxidase-tagged antibody (1:2000; Roche) in place of the anti-digoxigenin antibody in Step B.8. Substitute a PBS rinse for the NMT rinse in Step B.10. To visualize the peroxidase tag, substitute Vector VIP (Vector Laboratories, one drop of each kit component per 3.3 ml of PBS) for the NBT/BCIP solution in Step B.11. Rinse in PBS rather than in TE as shown in Step B.12.

G.2. *Double ISH.* Perform the ISH exactly as described in sections "Prehybridization" and "Hybridization." Add the FITC-riboprobe with the digoxigenin–riboprobe in Step D.1. Add sheep anti-FITC peroxidase-tagged antibody (1:2000) along with any other primary antibodies in Step F.4. After Step F.5, insert the following steps:

a. Incubate in biotin–tyramide at 1:75 in supplied diluent (Perkin-Elmer, Boston, MA) for 10 min in sterile 24-well tissue culture dish.

b. Transfer to mesh-well dish and rinse 3 × 5 min in TBS.
c. Incubate in streptavidin Cy3 at 1:200 (Jackson, West Grove, PA) plus any other secondaries as indicated in Step F.6.
d. Proceed with rest of protocol from Steps F.6 to F.13.

ACKNOWLEDGMENTS. This work was supported by grants HL 074011 and HL 28785 from the National Institutes of Health, Heart Lung and Blood Institute to P.G.G.

REFERENCES

Baldwin, T. J., Tsaur, M. L., Lopez, G. A., Jan, Y. N., and Jan, L. Y., 1991, Characterization of a mammalian cDNA for an inactivating voltage-sensitive K^+ channel, *Neuron* **7:**471–483.

Bellocchio, E. E., Hu, H., Pohorille, A., Chan, J., Pickel, V. M., and Edwards, R. H., 1998, The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission, *J. Neurosci.* **18:**8648–8659.

Bloch, B., Popovici, T., Chouham, S., and Kowalski, C., 1986a, Detection of the mRNA coding for enkephalin precursor in the rat brain and adrenal by using an "in situ" hybridization procedure, *Neurosci. Lett.* **64:**29–34.

Bloch, B., Popovici, T., Le Guellec, D., Normand, E., Chouham, S., Guitteny, A. F., and Bohlen, P., 1986b, In situ hybridization histochemistry for the analysis of gene expression in the endocrine and central nervous system tissues: a 3-year experience, *J. Neurosci. Res.* **16:**183–200.

Brysch, W., Creutzfeldt, O. D., Luno, K., Schlingensiepen, R., and Schlingensiepen, K. H., 1991, Regional and temporal expression of sodium channel messenger RNAs in the rat brain during development, *Exp. Brain Res.* **86:**562–567.

Chesselet, M. F., 1990, *In Situ Hybridization Histochemistry*, Boston: CRC Press.

Chesselet, M. F., Weiss, L., Wuenschell, C., Tobin, A. J., and Affolter, H. U., 1987, Comparative distribution of mRNAs for glutamic acid decarboxylase, tyrosine hydroxylase, and tachykinins in the basal ganglia: an in situ hybridization study in the rodent brain, *J. Comp. Neurol.* **262:**125–140.

Clavel, C., Binninger, I., Boutterin, M. C., Polette, M., and Birembaut, P., 1991, Comparison of four non-radioactive and 35S-based methods for the detection of human papillomavirus DNA by in situ hybridization, *J. Virol. Methods* **33:**253–266.

Darby, I. A., 2000, *In Situ Hybridization Protocols*, 2nd ed., Totowa, NJ: Humana Press.

Esclapez, M., Tillakaratne, N. J., Kaufman, D. L., Tobin, A. J., and Houser, C. R., 1994, Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms, *J. Neurosci.* **14:**1834–1855.

Fremeau, R. T., Voglmaier, S., Seal, R. P., and Edwards, R. H., 2004, VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate, *Trends Neurosci.* **27:**98–103.

Gall, J. G., and Pardue, M. L., 1969, Formation and detection of RNA-DNA hybrid molecules in cytological preparations, *Proc. Natl. Acad. Sci. U. S. A.* **63:**378–383.

Goldman, D., Simmons, D., Swanson, L. W., Patrick, J., and Heinemann, S., 1986, Mapping of brain areas expressing RNA homologous to two different acetylcholine receptor alpha-subunit cDNAs, *Proc. Natl. Acad. Sci. U.S.A.* **83:**4076–4080.

Guyenet, P. G., Stornetta, R. L., Weston, M. C., McQuiston, T., and Simmons, J. R., 2004, Detection of amino acid and peptide transmitters in physiologically identified brainstem cardiorespiratory neurons, *Auton. Neurosci.* **114:**1–10.

Hisano, S., Sawada, K., Kawano, M., Kanemoto, M., Xiong, G. X., Mogi, K., Sakata-Haga, H., Takeda, J., Fukui, Y., and Nogami, H., 2002, Expression of inorganic phosphate/vesicular

glutamate transporters (BNPI/VGLUT1 and DNPI/VGLUT2) in the cerebellum and precerebellar nuclei of the rat, *Mol. Brain. Res.* **107:**23–31.

Hoefler, H., Childers, H., Montminy, M. R., Lechan, R. M., Goodman, R. H., and Wolfe, H. J., 1986, In situ hybridization methods for the detection of somatostatin mRNA in tissue sections using antisense RNA probes, *Histochem. J.* **18:**597–604.

Hwang, P. M., Glatt, C. E., Bredt, D. S., Yellen, G., and Snyder, S. H., 1992, A novel K+ channel with unique localizations in mammalian brain: molecular cloning and characterization, *Neuron* **8:**473–481.

John, H. A., Birnstiel, M. L., and Jones, K. W., 1969, RNA-DNA hybrids at the cytological level, *Nature* **223:**582–587.

Johnson, A. D., Peoples, J., Stornetta, R. L., and Van Bockstaele, E. J., 2002, Opioid circuits originating from the nucleus paragigantocellularis and their potential role in opiate withdrawal, *Brain Res.* **955:**72–84.

Julien, J. F., Legay, F., Dumas, S., Tappaz, M., and Mallet, J., 1987, Molecular cloning, expression and in situ hybridization of rat brain glutamic acid decarboxylase messenger RNA, *Neurosci. Lett.* **73:**173–180.

Kreft, S., Zajc-Kreft, K., Zivin, M., Sket, D., and Grubic, Z., 1996, Application of the nonradioactive in situ hybridization for the localization of acetylcholinesterase mRNA in the central nervous system of the rat; comparison to the radioactive technique, *Pflugers Arch.* **431:**R309–R310.

Lanaud, P., Popovici, T., Normand, E., Lemoine, C., Bloch, B., and Roques, B. P., 1989, Distribution of CCK mRNA in particular regions (hippocampus, periaqueductal grey and thalamus) of the rat by in situ hybridization, *Neurosci. Lett.* **104:**38–42.

Lenz, S., Perney, T. M., Qin, Y., Robbins, E., and Chesselet, M. F., 1994, GABA-ergic interneurons of the striatum express the Shaw-like potassium channel Kv3.1, *Synapse* **18:**55–66.

Lewis, M. E., and Baldino, F., 1990, Probes for in situ hybridization histochemistry, In: Chesselet, M. F. (ed.), *In Situ Hybridization Histochemistry*, Boca Raton, FL: CRC Press, pp. 1–21.

Lewis, M. E., Sherman, T. G., and Watson, S. J., 1985, In situ hybridization histochemistry with synthetic oligonucleotides: strategies and methods, *Peptides* **6**(Suppl. 2)**:**75–87.

Malherbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J. G., and Mohler, H., 1990, Functional expression and sites of gene transcription of a novel alpha subunit of the GABAA receptor in rat brain, *FEBS Lett.* **260:**261–265.

McKinnon, D., 1989, Isolation of a cDNA clone coding for a putative second potassium channel indicates the existence of a gene family, *J. Biol. Chem.* **264:**8230–8236.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R., 1984, Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter, *Nucleic Acids Res.* **12:**7035–7056.

Mezey, E., 1989, Phenylethanolamine N-methyltransferase-containing neurons in the limbic system of the young rat, *Proc. Natl. Acad. Sci. U. S. A.* **86:**347–351.

Mitchell, V., Gambiez, A., and Beauvillain, J. C., 1993, Fine-structural localization of proenkephalin mRNAs in the hypothalamic magnocellular dorsal nucleus of the guinea pig: a comparison of radioisotopic and enzymatic in situ hybridization methods at the light- and electron-microscopic levels, *Cell Tissue Res.* **274:**219–228.

Park, J. S., Kurman, R. J., Kessis, T. D., and Shah, K. V., 1991, Comparison of peroxidase-labeled DNA probes with radioactive RNA probes for detection of human papillomaviruses by in situ hybridization in paraffin sections, *Mod. Pathol.* **4:**81–85.

Pelletier, G., Liao, N., Follea, N., and Govindan, M. V., 1988, Distribution of estrogen receptors in the rat pituitary as studied by in situ hybridization, *Mol. Cell. Endocrinol.* **56:**29–33.

Perney, T. M., Marshall, J., Martin, K. A., Hockfield, S., and Kaczmarek, L. K., 1992, Expression of the mRNAs for the Kv3.1 potassium channel gene in the adult and developing rat brain, *J. Neurophysiol.* **68:**756–766.

Pochet, R., Brocas, H., Vassart, G., Toubeau, G., Seo, H., Refetoff, S., Dumont, J. E., and Pasteels, J. L., 1981, Radioautographic localization of prolactin messenger RNA on histological sections by in situ hybridization, *Brain Res.* **211:**433–438.

Poyatos, I., Ponce, J., Aragon, C., Gimenez, C., and Zafra, F., 1997, The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons, *Mol. Brain Res.* **49:** 63–70.

Rogers, S. W., Hughes, T. E., Hollmann, M., Gasic, G. P., Deneris, E. S., and Heinemann, S., 1991, The characterization and localization of the glutamate receptor subunit GluR1 in the rat brain, *J. Neurosci.* **11:**2713–2724.

Rudy, B., Kentros, C., Weiser, M., Fruhling, D., Serodio, P., Vega-Saenz, D. M., Ellisman, M. H., Pollock, J. A., and Baker, H., 1992, Region-specific expression of a K+ channel gene in brain, *Proc. Natl. Acad. Sci. U. S. A.* **89:**4603–4607.

Schmued, L. C., and Fallon, J. H., 1986, Fluoro-gold: a new fluorescent retrograde axonal tracer with numerous unique properties, *Brain Res.* **377:**147–154.

Schreihofer, A.M., Stornetta, R.L., and Guyenet, P.G., 1999, Evidence for glycinergic respiratory neurons: Bötzinger neurons express mRNA for glycenergic transporter 2, *J. Comp. Neurol.* **407**:583–597.

Seroogy, K., Schalling, M., Brene, S., Dagerlind, A., Chai, S. Y., Hokfelt, T., Persson, H., Brownstein, M., Huan, R., Dixon, J., Filer, D., Schlessinger, D., and Goldstein, M., 1989, Cholecystokinin and tyrosine hydroxylase messenger RNAs in neurons of rat mesencephalon: peptide/monoamine coexistence studies using in situ hybridization combined with immunocytochemistry, *Exp. Brain Res.* **74:**149–162.

Shivers, B. D., Schachter, B. S., and Pfaff, D. W., 1986, In Situ hybridization for the study of gene expression in the brain, *Meth. Enzymol.* **124**: 497–510.

Siegel, R. E., and Young III, W. S., 1985, Detection of preprocholecystokinin and preproenkephalin A mRNAs in rat brain by hybridization histochemistry using complementary RNA probes, *Neuropeptides* **6:**573–580.

Stornetta, R. L., Akey, P. J., and Guyenet, P. G., 1999, Location and electrophysiological characterization of rostral medullary adrenergic neurons that contain neuropeptide Y mRNA in rat medulla, *J. Comp. Neurol.* **415:**482–500.

Stornetta, R. L., and Guyenet, P. G., 1999, Distribution of glutamic acid decarboxylase mRNA containing neurons in rat medulla projecting to thoracic spinal cord in relation to monoaminergic brainstem neurons, *J. Comp. Neurol.* **407:**367–380.

Stornetta, R. L., McQuiston, T. J., and Guyenet, P. G., 2004, GABAergic and glycinergic presympathetic neurons of rat medulla oblongata identified by retrograde transport of pseudorabies virus and in situ hybridization, *J. Comp. Neurol.* **479:**257–270.

Stornetta, R. L., Sevigny, C. P., and Guyenet, P. G., 2003, Inspiratory augmenting bulbospinal neurons express both glutamatergic and enkephalinergic phenotypes, *J. Comp. Neurol.* **455:**113–124.

Stornetta, R. L., Sevigny, C. P., Schreihofer, A. M., Rosin, D. L., and Guyenet, P. G., 2002, Vesicular glutamate transporter DNPI/GLUT2 is expressed by both C1 adrenergic and nonaminergic presympathetic vasomotor neurons of the rat medulla, *J. Comp. Neurol.* **444:**207–220.

Stornetta, R.L., Schreihofer, A.M., Pelaez, N.M., Sevigny, C.P., and Guyenet, P.G., 2001, Preproenkephalin mRNA is expressed by C1 and non-C1 barosensitive bulbospinal neurons in the rostral ventrolateral medulla of the rat, *J. Comp. Neurol.* **435**:111–126.

Surmeier, D. J., Eberwine, J., Wilson, C. J., Cao, Y., Stefani, A., and Kitai, S. T., 1992, Dopamine receptor subtypes colocalize in rat striatonigral neurons, *Proc. Natl. Acad. Sci. U. S. A.* **89:**10178–10182.

Terenghi, G., Polak, J. M., Hamid, Q., O'Brien, E., Denny, P., Legon, S., Dixon, J., Minth, C. D., Palay, S. L., Yasargil, G., and Chan-Palay, V., 1987, Localization of neuropeptide Y mRNA in neurons of human cerebral cortex by means of in situ hybridization with a complementary RNA probe, *Proc. Natl. Acad. Sci. U. S. A.* **84:**7315–7318.

Valentino, K. L., Eberwine, J. H., and Barchas, J. D., 1987, *In Situ Hybridization: Applications to Neurobiology*, New York: Oxford University Press.

Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E. S., Swanson, L. W., Heinemann, S., and Patrick, J., 1988, Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor, *Science* **240:**330–334.

Wang, H., Kunkel, D. D., Schwartzkroin, P. A., and Tempel, B. L., 1994, Localization of Kv1.1 and Kv1.2, two K channel proteins, to synaptic terminals, somata, and dendrites in the mouse brain, *J. Neurosci.* **14:**4588–4599.

Wilkinson, D. G., 1998, *In Situ Hybridization: A Practical Approach,* 2nd ed., Oxford: Oxford University Press.

Wisden, W., and Morris, B. J., 1994, *In Situ Hybridization Protocols for the Brain,* London: Academic Press, Harcourt Brace &Co.

Wisden, W., Morris, B. J., Darlison, M. G., Hunt, S. P., and Barnard, E. A., 1988, Distinct GABAA receptor alpha subunit mRNAs show differential patterns of expression in bovine brain, *Neuron* **1:**937–947.

Wuenschell, C. W., Fisher, R. S., Kaufman, D. L., and Tobin, A. J., 1986, In situ hybridization to localize mRNA encoding the neurotransmitter synthetic enzyme glutamate decarboxylase in mouse cerebellum, *Proc. Natl. Acad. Sci. U. S. A.* **83:**6193–6197.

Young III, W. S., 1990, In situ hybridization histochemistry, In: Björklund, A., Hökfelt, T., Wouterlood, F. G., and Van den Pol, A. N. (eds.), *Analysis of Neuronal Microcircuits and Synaptic Interactions,* Amsterdam: Elsevier.