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Modulation of tyrosine hydroxylase gene expression in the central nervous system visualized by *in situ* hybridization

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ABSTRACT **A rat tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] cDNA probe was used for *in situ* hybridization studies on histological sections through the locus coeruleus, substantia nigra, and the ventral tegmental area of the rat brain. Experimental conditions were established that yielded no background and no signal when pBR322 was used as a control probe. Using the tyrosine hydroxylase probe, we ascertained the specificity of the labeling over catecholaminergic cells by denervation experiments and comparison of the hybridization pattern with that of immunoreactivity. The use of ^{35}S-labeled probe enabled the hybridization signal to be resolved at the cellular level. A single injection of reserpine into the rat led to an increase of the intensity of the autoradiographic signal over the locus coeruleus area, confirming an RNA gel blot analysis. The potential of *in situ* hybridization to analyze patterns of modulation of gene activity as a result of nervous activity is discussed.**

Catecholaminergic cells of the central nervous system are found in small nuclei, mostly in the brain stem and mesencephalon (1, 2). In spite of their paucity (probably $<5 \times 10^4$ cells) these neurons send projections to nearly every region of the brain and play a crucial role in its function. Tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2], the rate-limiting enzyme in catecholamine synthesis, has been intensively investigated, and its expression has been found to be controlled by a variety of external factors, both in the developing and adult organism (3, 4). For instance, prolonged stimulation of TyrOHase activity has been observed following increased neuronal activity (5, 6). These long-term changes result from a rise in TyrOHase mRNA levels, most probably due to an increase in TyrOHase gene transcription (7, 8).

In the last decade, the use of TyrOHase antibodies has been instrumental in revealing the organization of various adrenergic, noradrenergic, and dopaminergic cell clusters and pathways in the brain. In the near future, it can confidently be predicted that the histochemical approach will greatly benefit from the availability of cloned cDNA probes. We report here the direct visualization of gene activity and its modulation on rat brain sections at the level of the locus coeruleus, the ventral tegmental area, and the substantia nigra. The complementarity of this approach with immunohistochemistry to analyze brain function and plasticity is discussed.

MATERIALS AND METHODS

Preparation of Probes. The construction and characterization of the recombinant pTH51 clone in plasmid pBR322 have been described (9, 10). The 1758-base-pair insert containing the complete coding and 3'-untranslated sequences was purified by electroelution after agarose gel electrophoresis and labeled by nick-translation (11) with ^{35}S-substituted adenosine and cytidine 5'-[α-thio]-triphosphates (Amersham) to specific activities of $1–3 \times 10^8$ dpm/μg. The average size of the nick-translated fragments was $\approx$500 base pairs. Control pBR322 DNA was nick-translated and used as described for pTH51.

Tissue Preparation. Male Sprague–Dawley rats (200–250 g) were used in these experiments. One group of rats received a 4-μl unilateral injection of a 6-hydroxydopamine solution (2 μg/μl) (12). A second group was treated with a single dose of reserpine (10 mg/kg) as described (13). Control animals were injected with vehicle only. Rats treated with 6-hydroxydopamine and with reserpine were sacrificed 6 and 2 days after injection, respectively.

Treated and control animals were anesthetized with pentobarbital and perfused through the ascending aorta for 10 min with ice-cold 0.9% NaCl followed by ice-cold 0.1 M potassium phosphate buffer at pH 7.4, containing 4% (wt/vol) paraformaldehyde for 10 min. The brains were removed, post-fixed in the same fixative for 1 hr at 4°C, soaked for an additional 18 hr in phosphate buffer containing 15% (wt/vol) sucrose, and rapidly frozen in isopentane at −60°C. Sections (10 μm) of appropriate areas were cut, thaw-mounted onto gelatin-coated slides, and stored at −70°C until use.

***In Situ* Hybridization.** Experiments were carried out essentially as described (14) with some modifications. Briefly, sections were prehybridized for 1 hr at room temperature in a solution containing 4× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) and 1× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin). The slides were then rinsed in three baths of absolute ethanol and air dried.

About 0.5 ng of nick-translated probe in 10 μl of hybridization solution [50% (vol/vol) formamide, 4× SSC, 1× Denhardt, 1% Sarcosyl, 10 mM dithiothreitol, 0.1 M potassium phosphate at pH 7.4, 100 ng of pBR322, 100 ng of yeast tRNA, and 100 ng of herring sperm DNA] was boiled 5 min, applied onto each section, and sealed under a coverslip. Hybridization was allowed to proceed for 18 hr at 42°C. Coverslips were then carefully removed, and the slides were rinsed at 42°C twice for 30 min in 1× SSC and twice for 30 min in 0.1× SSC before being dehydrated in absolute ethanol and dried at room temperature.

Autoradiograms of tissue were generated by apposition of ^{35}S-labeled sections to X-Omat film (Kodak) or by dipping in Ilford-K5 emulsion. After appropriate times of exposure,

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Abbreviation: TyrOHase, tyrosine hydroxylase.

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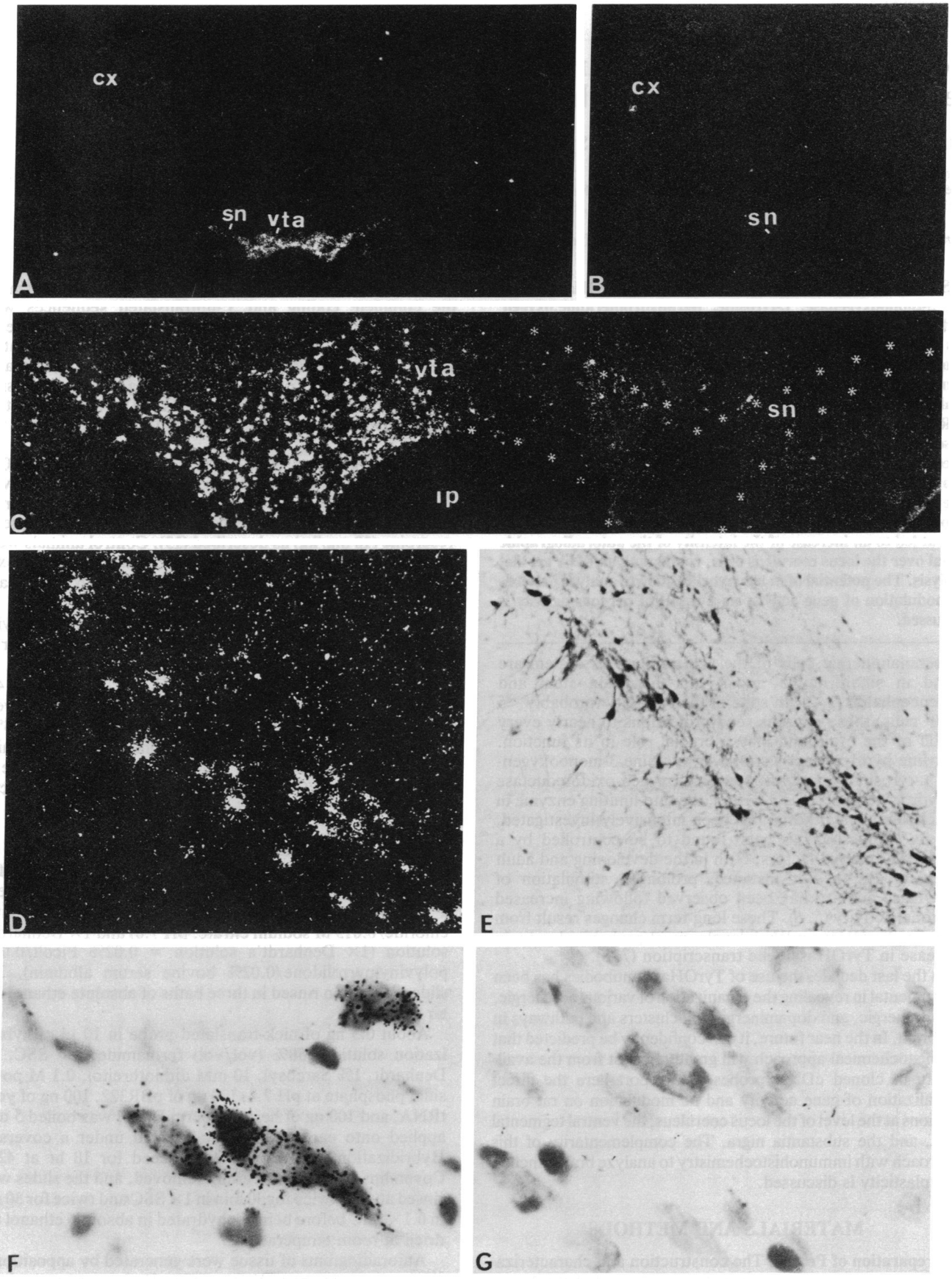

FIG. 1. *In situ* hybridization to rat brain sections at the level of the substantia nigra (sn) and the ventral tegmental area (vta) using a ^{35}S-labeled TyrOHase cDNA probe. (*A* and *B*) Sections exposed in direct contact with x-ray film. An intense labeling is visible over sn and vta (*A*). No signal is observed over these areas or cerebral cortex (cx) with a control pBR322 probe (*B*). (*C*) Dark-field view of an emulsion-coated section after injection of 6-hydroxydopamine in the right sn. Note the total disappearance of the signal on the injected side. ip, Interpeduncular nucleus.

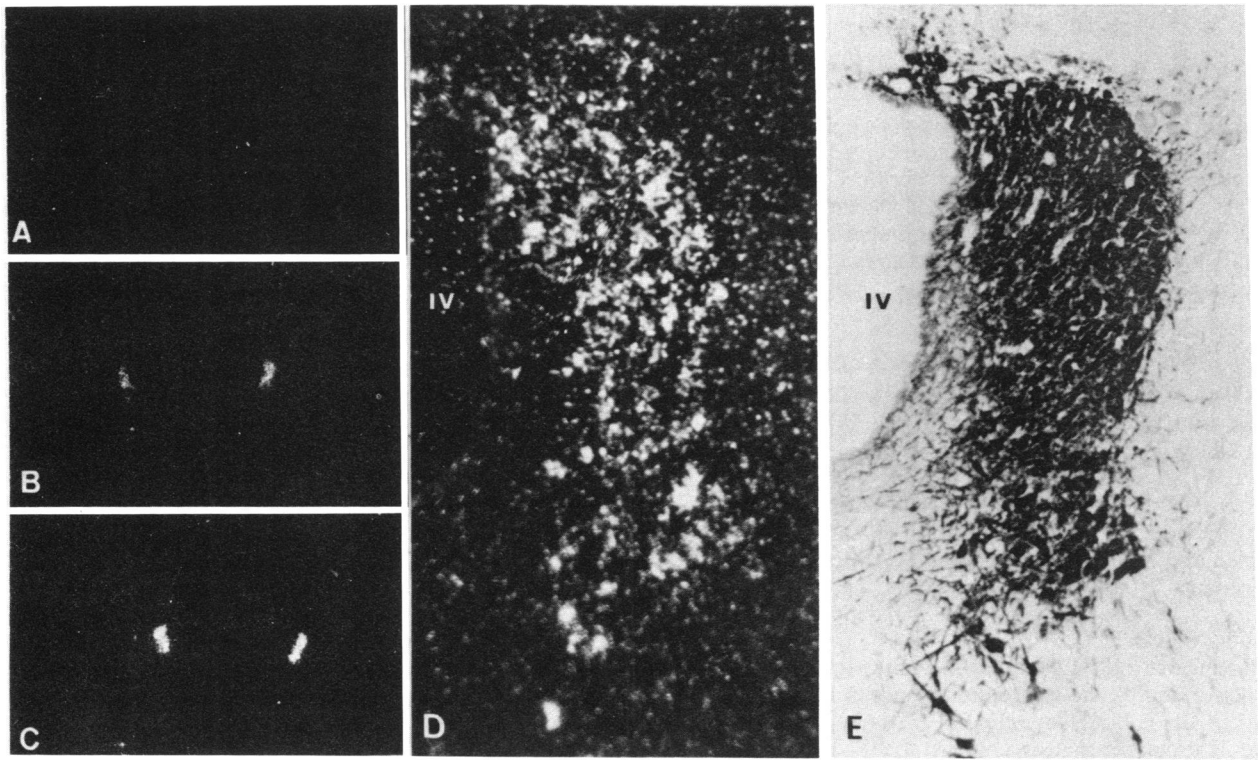

FIG. 2. *In situ* hybridization to rat brain sections at the level of the locus coeruleus using a ^{35}S-labeled TyrOHase cDNA probe. (*A*, *B*, and *C*) Sections exposed in direct contact with x-ray film. No signal is observed with a control pBR322 probe (*A*). *B* and *C* are sections from a normal and a reserpine-treated rat, respectively, hybridized in parallel with the TyrOHase probe. (*D* and *E*) Dark-field/bright-field comparative views of adjacent sections subjected to hybridization with the TyrOHase probe (*D*) or stained with TyrOHase antibody (*E*), respectively. IV, Fourth ventricle. Exposure times were 2–4 days. (Magnifications: *A*, *B*, and *C*, ×10; *D* and *E*, ×110.)

films and emulsions were developed, and tissue sections were stained with cresyl violet.

Immunochemistry. Two anti-TyrOHase sera were used indifferently. The first, raised against TyrOHase purified from bovine adrenal glands, has already been described (15). The second was raised against a β-galactosidase-rat TyrOHase fusion protein using the pEX plasmid expression vector (16) (Ph. Kahn, A.B., and J.M., unpublished data). Both antibodies were produced in rabbits.

Tissue sections were post-fixed for 15 min in a 8% (wt/vol) paraformaldehyde solution and treated as described (17), according to the double-bridge peroxidase-antiperoxidase method developed by Sternberger *et al.* (18).

RESULTS

We first examined the hybridization pattern of the TyrOHase cDNA probe on a transverse section through the dopaminergic area of the rat mesencephalon. Autoradiography with x-ray films in contact with the sections revealed an intense labeling in the ventral tegmental area and the substantia nigra (Fig. 1*A*).

Experiments were carried out to analyze the specificity of the hybridization pattern. First, a section adjacent to that shown in Fig. 1*A* was hybridized with a control pBR322 probe, and no signal was observed (Fig. 1*B*). Second, a selective unilateral destruction of dopaminergic neurons was performed by local injection of 6-hydroxydopamine to confirm that the specific labeling resulting from *in situ* hybridization was associated with these cells. As can be seen in Fig. 1*C*, this treatment led to a total disappearance of the signal on the injected side.

To obtain a resolution of the labeling at the cellular level, the slides were coated with an emulsion. Dark-field microscopic examination revealed a great number of labeled cell bodies in the ventral tegmental area and the pars compacta of the substantia nigra (Fig. 1*D*). In the caudal pars reticulata and lateralis of the substantia nigra, smaller groups of cells or single cells were also specifically labeled. For comparison, a section adjacent to that shown in Fig. 1*E* was stained with TyrOHase antibodies. Inspection of Fig. 1 *D* and *E* reveals a similar pattern of reactivity. The distribution of these patterns of reactivity is in agreement with data concerning dopaminergic cell localization in these areas (19). Bright-field analysis at a higher magnification clearly indicates that the silver grains are associated with discrete cell bodies (Fig. 1*F*) that display no hybridization with the control probe (Fig. 1*G*).

A second series of experiments were performed on brain sections through the noradrenergic neurons of the locus coeruleus. Fig. 2 *A* and *B* permit the comparison of the hybridization pattern with the control and TyrOHase probe, respectively, after x-ray autoradiography. A specific labeling is clearly associated with locus coeruleus cells.

The microscopic distribution of positively reacting cells in the locus coeruleus was examined on an emulsion-coated slide. Fig. 2*D* shows the labeling of a cluster of cells along the fourth ventricle and demonstrates that the intensity of the labeling is heterogeneous. This pattern matches that of the

(*D* and *E*) Dark-field/bright-field comparative views of adjacent sections subjected to hybridization with the TyrOHase probe (*D*) or stained with TyrOHase antibody (*E*), respectively. (*F* and *G*) Bright-field views of sn cells. Note the accumulation of the grains over dopaminergic neurons (*F*). Again, no labeling is detectable with the control probe (*G*). Exposure times were 2–4 days. (Magnifications: *A* and *B*, ×10; *C*, ×80; *D* and *E*, ×120; *F* and *G*, ×830.)

immunoreactivity with TyrOHase antibodies on an adjacent section (Fig. 2*E*).

To determine whether the intensity of the labeling over locus coeruleus paralleled the amount of TyrOHase mRNA present in this structure, hybridizations were also performed on brain sections from rats that had received a single dose of reserpine 2 days before they were sacrificed. Fig. 2*C* clearly demonstrates that the intensity of the labeling over the locus coeruleus is greatly increased. Quantitative autoradiographic analysis indicated that, over the locus coeruleus area, the intensity is ≈4-fold higher than in the control.

DISCUSSION

In this study we have used *in situ* hybridization on tissue sections to visualize TyrOHase gene activity in the central nervous system. The autoradiographic signal obtained on locus coeruleus sections of reserpine-treated rats increased quite significantly relative to control animals. This analysis complements data we have obtained from RNA gel blot experiments (8) and demonstrates that the intensity of the labeling correlates well with the abundance of the corresponding transcripts.

Labeling of a cDNA encoding the entire TyrOHase mRNA was performed by the conventional nick-translation method using ^{35}S-labeled nucleotides. The ^{35}S isotope constitutes a convenient marker that, in contrast to ^{32}P, provides resolution at the cellular level and avoids the long exposure times that are required with ^{3}H-labeled probes. Tissues were fixed by perfusion with paraformaldehyde, which allows both hybridization and immunohistochemical analysis.

The specificity of hybridization with the TyrOHase probe was analyzed by several criteria, taking advantage of the wealth of available information concerning catecholaminergic neurons. First of all, a control plasmid that did not contain TyrOHase sequences yielded no signal. Second, a local injection of 6-hydroxydopamine, a drug which specifically destroys catecholaminergic neurons, abolished the labeling in the substantia nigra. Third, *in situ* hybridization and immunochemical reactivities of TyrOHase on adjacent sections displayed a similar general pattern (Fig. 1 *D* and *E* and Fig. 2 *D* and *E*). However, a more detailed analysis may well reveal that the relative intensities of the two labelings do not always correlate. Such discrepancies are to be expected, considering that TyrOHase molecules are transported along axons. The cell bodies are very probably heterogeneous with respect to the amount of enzyme they contain, depending upon the terminal area to which TyrOHase is transported. In support of this hypothesis, we have shown that adrenal cells, where the enzyme is not transported, contain approximately four times less TyrOHase mRNA per unit of enzyme activity than locus coeruleus or substantia nigra (8).

The modulation of gene expression could be directly assessed on tissue sections after reserpine treatment. This drug produces an increase of TyrOHase activity in adrenal and superior cervical ganglia, which was shown to be abolished by interruption of the afferent nerves, and was, therefore, referred to as trans-synaptic induction (5, 20, 21). In the locus coeruleus, reserpine induces a similar increase in enzyme activity (6, 22, 23), which reflects an increase in TyrOHase mRNA levels with a maximal 4-fold stimulation relative to control animals 2 days after reserpine injection (8). This increase in TyrOHase mRNA concentration was also clearly revealed by *in situ* hybridization as shown in Fig. 2 *B* and *C*. Quantitative analysis of the autoradiogram indicates a similar ≈4-fold increase of the signal after reserpine treatment (result not shown). This result provides further evidence that the autoradiographic signal corresponds to a specific hybridization of the probe with TyrOHase mRNA.

Hybridization histochemistry will be essential to analyze induction in other catecholaminergic nuclei that contain too few cells to be individually amenable to RNA gel blot experiments. Furthermore, by allowing a direct measurement of the activity of the TyrOHase gene in a given cell body, new subpopulations of catecholaminergic neurons might be revealed. This approach will greatly facilitate physiological and pharmacological analyses that have been hampered, at the protein level, by the complex intermingling of TyrOHase-immuno-positive fibers originating from various cell bodies.

A detailed analysis of TyrOHase mRNA expression during development and in the adult should also prove to be very instructive in view of the plasticity of neurotransmitter-related phenotypic expression. Original studies carried out in the peripheral nervous system revealed that the noradrenergic phenotype can convert into the cholinergic phenotype depending upon the environment of these neurons (24). Moreover, dual-function noradrenergic–cholinergic neurons have been identified (25). Adult cholinergic neurons *in vivo* can be induced to express adrenergic characteristics (26). A plasticity also occurs in the central nervous system and in some instances represents an integral part of the development of the animal (4). This plasticity, which could play an important role in the adaptation of the system to perturbation, can now be approached at the molecular level. Neurons may maintain, in an "open chromatin conformation" (27), sets of genes pertaining to various transmitter phenotypes. If so, it is conceivable that the corresponding mRNA are continuously transcribed but not translated. In other words, the transcription of an *a priori* unexpected mRNA could be an indication of the plasticity of a given phenotype, and *in situ* hybridization will be invaluable in this analysis.

Clearly, hybridization histochemistry has become a crucial tool for exploring the molecular basis of brain function.

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