Expression of Tyrosine Hydroxylase in Neurons of Cultured Cerebral Cortex: Evidence for Phenotypic Plasticity in Neurons of the CNS

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***In vivo*, neurons of the cerebral cortex of rat embryos did not stain with antibodies to the catecholamine (CA) biosynthetic enzyme tyrosine hydroxylase (TH) even when examined using a highly sensitive technique for radioimmuno-cytochemistry. However, when embryonic day (E) 13 cortex was grown 1 d in culture, several thousand cells expressed immunoreactive and catalytically active TH. All TH cells simultaneously labeled with the neuronal enzyme, neuronal specific enolase, indicating that the TH was exclusively localized in neurons. Moreover, all TH neurons were postmitotic since they did not incorporate ³H-thymidine. With time in culture, the number of TH cells selectively declined from nearly 3000 cells at 2 d to several cells at 14 d. Similarly, the number of neurons competent to express TH in culture declined with advancing age of the donor embryo. Thus, by E18, very few cortical neurons had the capacity to express TH.**

We conclude that during a critical period of development, postmitotic cerebral cortical neurons can express catecholamine traits *in vitro* but not *in vivo*. Thus, the neurotransmitter phenotype of certain classes of central neurons is not fixed but can be influenced by epigenetic factors found in their environment, thereby providing evidence of phenotypic plasticity in the central nervous system (CNS).

It is now well established that the neurotransmitter phenotype of neurons of the peripheral nervous system (PNS), once held to be immutable, is in fact extremely plastic. For example, the biochemical differentiation of cells of the neural crest into adrenergic neurons of sympathetic ganglia or cholinergic parasympathetic neurons depends greatly on factors encountered in the environment during and after their embryonic migration (LeDouarin, 1980). Moreover, these factors continue to modulate phenotypic expression even after neural crest cells have withdrawn from the mitotic cycle and expressed differentiated properties (Landis and Keefe, 1973; Bunge et al., 1978; Patterson, 1978). Thus, under appropriate conditions, fully differentiated sympathetic neurons can express cholinergic traits (Bunge et al., 1978; Patterson, 1978) and, conversely, parasympathetic neurons can express adrenergic traits (Landis et al., 1983; Iacovitti et al., 1985a; Teitelman et al., 1985).

It is not known whether neurons in the central nervous system (CNS) share comparable degrees of biochemical plasticity. That they may was suggested by a recent study in the retina (Schnitzer and Rusoff, 1984) as well as by our own observation that if the cerebral cortex of embryonal rats was dissociated, grown in culture and stained with antibodies to the catecholamine (CA) biosynthetic enzyme tyrosine hydroxylase (TH), many cells in the culture contained the enzyme (Iacovitti et al., 1985b). The observation was unexpected since it is well established that in the adult neurons which synthesize, store, and release CAs are not contained within the cerebral cortex but rather reside within specific nuclei of the brain stem (Ungerstedt, 1971). The unexpected appearance of TH in cells which do not normally contain it raises the possibility that the neurons of the cerebral cortex may be influenced by epigenetic factors present in their environment.

In the present study we therefore sought to determine whether the expression of the TH phenotype in cells of the embryonal cerebral cortex is restricted to neurons, whether such neurons have completed their cell replication, whether the expression of the enzyme is transient or permanent, and whether it is influenced by the age at which the cells are removed from the developing rat. We shall demonstrate, immunocytochemically and biochemically, that postmitotic neurons of the cerebral cortex can express the CA enzyme TH in culture but not *in vivo*.

Materials and Methods

Tissue culture

Pregnant Sprague-Dawley rats were purchased from Hilltop Lab Animals (Scottsdale, Pa.) at 12 d gestation $\pm$ 12 hr fertilization day = embryonic day (E) 0. On E13, 14, 16, or 18, rats were anesthetized with pentobarbital, and the embryos removed. After dissection of the brain from the cranium, the cerebral cortices, excluding the anterior poles, were isolated. After the removal of the meninges, the cortical tissue was incubated in Ca^{2+} -, Mg^{2+} -free Hank's balanced salt solution (CMF-HBSS) for 10 min at 37°C in a clinical rotator (minimum setting). The incubation mixture was replaced with a 0.01% trypsin solution (in CMF-HBSS), and cortices incubated for another 10 min, rinsed twice in Leibovitz medium (L-15), and placed in culture medium containing Dulbecco's minimum essential medium, 10% fetal calf serum (Irvine Scientific) glucose (6 mg/ml), glutamine (204 $\mu\text{g}/\text{ml}$), and penicillin/streptomycin (100 U/ml). The cells were dissociated by trituration through a reduced bore borosilicate glass pipette. The cell suspension was filtered through a sterile 15- μm Nitex filter and plated onto plastic (Aclar) culture dishes coated either with polymerized collagen or with polyornithine (precoated for 4 hr at room temperature at a concentration of 1.0 mg/ml in 15 mM borate buffer, pH 8.4). The cellular plating density corresponded to 2 cortices/dish (approximately 5×10^5 cells/dish). Cortical cells formed fewer cell aggregates on polyornithine than on collagen; hence, in radioautography experiments, which required that all cells be evenly coated with emulsion, cells were grown on polyornithine. In all other experimental manipulations, both substrata were used.

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Figure 1. Immunocytochemical localization of TH in cross sections of E14 rat brain. Note absence of TH labeling in cortical cells (*A*) even though CA cell bodies and processes are darkly stained in brain-stem nuclei (*B*).

Immunocytochemistry

Single label experiments. Rat embryos were removed at E13, 14, 16, and 18, and immersed in fixative solution (4% formaldehyde generated from paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2). The brains were removed, fixed for 1 hr, washed twice with 0.1 M phosphate

buffer, embedded in a 30% sucrose solution overnight, and serially sectioned at 16 μ m in a cryostat microtome. The sections were thaw-mounted onto glass slides precoated with a 1% gelatin solution.

Immunohistochemical staining was carried out using either the soluble peroxidase-antiperoxidase (PAP) method of Sternberger (1974) as described by Teitelman et al. (1978), or the highly sensitive 125 I-labeling

Figure 2. Immunocytochemical localization of TH in E14 rat cortex maintained 2 d *in vitro* (**A**). Note the beaded varicose processes elaborated by stained neurons (**B**, arrow).

technique described in the following section. The procedures for preparation of antibodies to TH and DBH and the criteria for establishing their specificity are described elsewhere (Joh and Ross, 1983; Teitelman et al., 1985). Antibodies were determined to be specific by their ability to precipitate enzyme activity from a crude tissue homogenate, by the production of a single immunoprecipitin arc when analyzed by immunodiffusion and immunoelectrophoresis against a crude antigen preparation, and by the highly sensitive technique of Western blotting. Antibodies to neuronal specific enolase (NSE) were purchased from Polysciences, Inc.

In experiments designed to study TH expression in cortical neurons *in vitro*, cultures incubated 1, 3, 5, 8, 10, and 14 d were rinsed extensively in Hank's balanced salt solution and then fixed for 60 min in the formaldehyde solution described above. Following fixation, cultures were stained by the PAP method as described by Iacovitti et al. (1981).

Double labeling. The procedure for labeling 2 antigens simultaneously (double label) have been described elsewhere (Pickel et al., 1986). Briefly, cultures were first incubated overnight with antibodies against TH (diluted 1:30,000). Such a high dilution of antibody was chosen in order to avoid nonspecific labeling of TH cells with the DAB used to localize the second antigen, NSE. Cultures were next incubated for 45 min with a 1 to 50 dilution [Tris buffered saline (TBS) + 1% goat serum] of a solution of donkey-antirabbit IgG labeled with 125 I (specific activity, 5–20 μ Ci/ μ g; Amersham). The following day the cultures were rinsed 6 times in TBS and exposed for 24 hr to a 1:500 dilution of antibodies against NSE. Finally the cultures were sequentially incubated with a 1:50 dilution of goat antirabbit IgG for 30 min and to a 1:100 dilution of PAP. The bound PAP complex was visualized by incubating the cultures with 0.013% DAB and 0.01% of hydrogen peroxide. After the

DAB reaction the cultures were air-dried overnight and dipped in Ilford L-4 photographic emulsion, air-dried and incubated in a light-tight box containing a drying agent (Drierite) at 4°C. After 1 week the cultures were developed for 3 min at 16–17°C in Kodak D-19 developer, rinsed in water, fixed in Kodak Ektaflo (diluted 1:3) for 8 min at 4°C, dehydrated and mounted in Permount.

Thymidine labeling

To determine whether cells in culture synthesize DNA, cortical cells were plated as described and after 1 hr media containing 0.05 μ Ci/ml of 3 H-thymidine (specific activity, 78.0 Ci/mmol) was added. After 1 d *in vitro*, cultures were fixed with 4% formaldehyde and processed for the immunohistochemical visualization of TH as well as the autoradiographic localization of incorporated thymidine. This double-labeling technique has been described previously (Teitelman et al., 1985). Thymidine-labeled cells were identified by the presence of silver grains over the nucleus.

TH assay

For biochemical determination of TH enzyme activity, cortical cultures were processed as described elsewhere (Iacovitti et al., 1981). Sister cultures were simultaneously stained with antibodies to TH and positively labeled cells were counted. It was estimated from cell counts that biochemical samples each contained approximately 20,000 TH cells. As controls for the assay, the adult rat substantia nigra, an area rich in TH neurons, was simultaneously homogenized and assayed using a modification of the method of Coyle (1972). All samples were run in duplicate

Figure 3. Cultures of E14 cortex maintained 2 d *in vitro* and sequentially stained with TH, visualized by radioautography, and NSE, visualized by the peroxidase-antiperoxidase technique. Note the presence of neurons which label only for NSE, seen as the brown precipitate of diaminobenzidine. Other cells, indicated by arrow, contain both TH and NSE since they label both with silver grains and diaminobenzidine (see inset).

in the presence and absence of the tyrosine substrate inhibitor, 3-iodotyrosine (2×10^{-4} M). Samples were pre-incubated for 5 min at 30°C with or without inhibitor before tyrosine was added to the assay mixture.

Results

TH in cerebral cortical neurons *in vivo*

Brains were removed from fetal rats on days E13, 14, or 16, serially sectioned, and processed for the immunocytochemical localization of TH using either the PAP method or the highly sensitive 125 I-labeling technique.

TH-immunoreactive cells were not seen in the cerebral cortex (Fig. 1A). However, neurons of the developing substantia nigra were intensely labeled (Fig. 1B), demonstrating that the absence of staining in cortex could not be attributed to a failure of the antibody to stain embryonic tissue. These findings, in agreement with others (Specht et al., 1981), indicate that in the midgestational embryo, as in the adult (Ungerstedt, 1971), neurons of the cerebral cortex do not contain detectable levels of immunoreactive TH.

Appearance of TH in neurons *in vitro*

In contrast to the situation *in vivo*, cultures of cerebral cortex obtained from rats on E13–14 contained a large population of cells which stained with antibodies to TH (Fig. 2A). Staining of cortical cells with TH was observed as early as 1 d *in vitro*. No cells stained with antibodies to DBH.

To further establish whether TH was expressed in neurons, cultures were processed for the simultaneous localization of TH and the neuronal marker, neuronal specific enolase (NSE). All TH-immunoreactive cortical cells also contained NSE (Fig. 3), indicating that TH was expressed exclusively in neurons of the

cerebral cortex and not in glia nor endothelium. Since those cells that labeled both with TH and NSE represented only a small fraction of the NSE-labeled cells in culture, TH expression was restricted to a particular subclass of cortical neurons. The neurons within this subclass, however, were morphologically heterogeneous; labeled neurons were stellate, bipolar, and pyramidal in shape. Many TH cells elaborated varicose processes (Fig. 2B).

Enzymatic activity of TH in cortical cultures

To determine if immunoreactive TH reflected the presence of catalytically active enzyme, TH activity was measured in cultures grown 2 d *in vitro*. Sister cultures were simultaneously

Table 1. TH activity levels in cerebral cortex grown 2 d *in vitro*

Tissue	3-Iodotyrosine pretreatment	CPM
Buffer blank	—	402
Buffer blank	+	413
Cultures of cerebral cortex	—	669
Cultures of cerebral cortex	+	356
Substantia nigra	—	29,936
Substantia nigra	+	437

In a typical experiment, 2-day old cultures of E14 cerebral cortex were pooled into samples, each containing approximately 20,000 TH immunoreactive neurons. Cultures were homogenized and assayed in the presence and absence of the competitive inhibitor, 3-iodotyrosine (2×10^{-4} M). As controls, homogenates of adult rat substantia nigra were assayed simultaneously.

Figure 4. Combined radioautography and immunocytochemistry in cultures of E14 cortex grown 1 d *in vitro* in the presence of ^3H -thymidine. Note that cells containing TH immunoreactivity do not have silver grains over their nuclei (arrow).

processed for immunocytochemistry and the number of neurons containing TH were counted.

In 3 separate experiments, 2-d-old cultures were pooled into samples each containing approximately 20,000 TH-positive neurons and were radioenzymatically assayed in the presence and in the absence of the competitive substrate inhibitor 3-iodotyrosine (Table 1). In all experiments, counts derived from cortical cultures were extremely low, never reaching levels greater than 400 cpm above blank. However, since even these low values were reduced to blank level by preincubating cortical homogenates with 3-iodotyrosine, they probably represent very low levels of TH catalytic activity. For comparison, homogenates of adult rat substantia nigra were simultaneously assayed. TH activity, which is very high in nigra, was also effectively eliminated when assayed in the presence of 3-iodotyrosine.

Relationship of expression of TH to cell cycle

The majority of cells in the rat cerebral cortex actively divide at E13 (Raedler and Sievers, 1976; Raedler and Raedler, 1978). To establish whether the cortical neurons expressing TH are still mitotic, cortices removed from rats at E13 were grown in media containing ^3H -thymidine. After 1 d, cultures were fixed and processed for the immunocytochemical localization of TH and radioautography.

TH-immunoreactive cortical cells did not incorporate ^3H -thymidine into their nuclei (Fig. 4). In contrast, silver grains were seen over the nuclei of the majority of cells lacking TH (Fig. 4), indicating that they had replicated while in culture. Thus, at E13, the neurons expressing TH in the cerebral cortex no longer divide.

Effects of time in culture on expression of TH

To assess whether the duration of time in culture influenced the phenotypic expression of TH in cortical neurons, cortices were removed from rat embryos at E13, plated and examined 2, 8, and 14 d later.

Despite the fact that the total number of neurons in the dish did not appear to decline with time in culture, the number of neurons expressing TH decreased dramatically (Table 2). Thus, at 2 d there were nearly 3000 TH neurons, at 8 d, fewer than a thousand TH neurons, and by 14 d, very few TH neurons were present, indicating a loss either in TH expression or cell viability with time in culture.

Effect of age of donor on expression of TH in cortical cultures

To establish whether the capacity of neurons to express TH was related to the embryonic age of the tissue, cerebral cortices were

Table 2. Decline in number of TH immunoreactive cortical cells with time in culture

Days <i>in vitro</i>	Number of TH cells/culture
2	2717 $\pm$ 841
8	893 $\pm$ 189
14	21 $\pm$ 8

Each number represents the mean $\pm$ SE of 4 cultures from 2 different experimental platings.

Table 3. Effect of *in vivo* age on the number of TH immunoreactive cortical cells in culture

<i>In vivo</i> age at time of explantation (E)	Number of TH cells/culture at 2 d <i>in vitro</i>
13–14	2444 $\pm$ 636
16	339 $\pm$ 121
18	21 $\pm$ 4

Each number represents the mean $\pm$ SE of 4 cultures from 2 different experimental platings.

removed from rats at E13, 16, and 18, plated and examined 2 d later. As seen in Table 3, the number of TH-immunoreactive neurons dramatically declined as the age of the donor embryo increased. Thus, cortical cultures derived from embryos at E13–14 contained several thousand TH-positive neurons, those from embryos at E16 several hundred, and those from embryos at E18 contained only a rare TH-immunoreactive cell. These results suggest that either cortical neurons have the capacity to express TH in culture only during a critical period in their development, or that mature cortex contains a substance(s) which inhibits the expression of TH.

To assess whether the cortex at E18 contained a substance(s) which might inhibit the expression of TH in the cortex of a younger animal, co-cultures were established consisting of cortical cells obtained from both young and old embryos. Cells from E18 rat cortex were dissociated and plated simultaneously with E14 cortical cells. Sister cultures from either E14 embryos or from E18 embryos were grown as controls. While in culture, it was not possible to distinguish between cells of different ages, co-cultures examined 2 and 8 d after plating showed no loss of TH neurons (Table 4) as compared with cultures containing only E14 cortical cells. This indicates that contact with or substances released by the mature cortex did not prevent the expression of TH in younger tissues and, therefore, cannot explain the failure of more mature cortical neurons to express TH. Quite to the contrary, there was a 2-fold increase in the number of TH-containing neurons in co-cultures of E14 and 18 cortex (Table 4), suggesting that factors present in co-culture promote an increase either in the number of cells expressing TH or in the viability of TH cells *in vitro*.

Discussion

We have found, in agreement with others (Specht et al., 1981), that the cerebral cortex of the embryonic rat is devoid of perikarya containing the CA biosynthetic enzyme TH. Yet if the cortex is removed from the embryo, dissociated and grown in culture, many of its cells express the enzyme. These TH-positive cells are neurons since they all contain the specific neuronal marker NSE. Moreover, the neurons which express TH do not synthesize DNA and are, therefore, postmitotic. The enzyme present in these neurons is both immunoreactive and catalytically active. The fact that DBH is not detected in cultures suggests that the neurons may have the capacity to synthesize dopamine but not other CA neurotransmitters. Whether or not these neurons synthesize, store, and/or release CAs is not known.

The appearance of a heretofore unexpressed neurotransmitter trait in postmitotic cortical neurons indicates that the neurotransmitter phenotype of these cells is not predetermined during development but can be epigenetically regulated. In the PNS, the environment is known to play a critical role in specifying neurotransmitter type (Bunge et al., 1978; Patterson, 1978; LeDouarin, 1980; Landis and Keefe, 1983; Iacovitti et al., 1985a; Teitelman et al., 1985). Our finding in cortex indicates that the same principles which govern neurotransmitter choice in peripheral neurons may also apply to certain classes of central neurons.

However, in contrast with the PNS where neurotransmitter phenotype remains labile for several weeks after cells have withdrawn from the cell cycle (Bunge et al., 1978; Patterson, 1978; Iacovitti et al., 1985a; Teitelman et al., 1985); in the cerebral cortex, the developmental window for phenotypic plasticity is much narrower. Thus, at E13, many cortical cells have the ca-

Table 4. Co-culture of E14 and E18 cortex increases the number of TH immunoreactive cortical cells

Cortex	Number of TH cells	
	2 d <i>in vitro</i>	8 d <i>in vitro</i>
E14	2335 ± 119	622 ± 239
E18	11 ± 7	0
E14 + E18	5016 ± 903	1385 ± 149

Each number represents the mean ± SE of 6 cultures from 2 separate experimental platings.

capacity to express TH in culture, but by E18 only an occasional TH competent cell remains. It is unlikely that the loss of this capacity reflects the presence of a substance(s) in maturing cortex which inhibit TH expression since the ability of E14 cortical cells to express TH is not only maintained when co-cultured with E18 cortex, but is substantially increased. Rather, it seems more likely that there is a critical period during embryogenesis of the cortex when neurons are competent to express a novel enzyme, such as TH, after which time their phenotype becomes fixed.

Even in those cortical neurons which are competent to express TH in culture, the expression of the enzyme appears to be short-lived. Thus, the number of TH-positive neurons gradually declines with time in culture. It is conceivable that the factors which signal TH expression differ from those that maintain it, with the latter decreasing over time *in vitro*. Indeed, the transient expression of neurotransmitter synthetic enzymes has been described in a number of neural (Cochard et al., 1978; Teitelman et al., 1978, 1981a; Jonakait et al., 1979, 1984; Schnitzer and Rusoff, 1984; Berger et al., 1985) and non-neural (Teitelman et al., 1981b, c) tissues. For example, islet cells of the pancreas contain TH only transiently before transformation into cells of another phenotypic characterization (Teitelman et al., 1981b, c). Cortical cells, in a similar fashion, may forfeit their expression of TH as they acquire traits of yet another neurotransmitter system. Alternatively, the disappearance of TH positive cortical neurons may result from the death of these cells over time in culture.

The fact that TH is seen in cortical neurons *in vitro* but not *in vivo* indicates that the environment provided in culture facilitates this expression. The source of these environmental signals, however, remains unknown. It is unlikely that serum factors are involved in the signaling process even though fetal calf serum is present in these experiments, since cultures established in a serum-free defined medium continue to contain abundant TH immunoreactive cells (unpublished observations). Alternatively, it is possible that CA differentiation is promoted in cortical neurons as a result of differences in the cellular microenvironment *in vivo* and *in vitro*. However, this too seems an unlikely explanation in light of the fact that when normal histiotypic relationships are maintained in a cortical transplant, cells still express TH (Smith et al., 1985). Taken together, these findings raise the possibility that environmental factors do not directly activate expression of the TH gene. Rather, it is possible that certain classes of cortical neurons have the intrinsic ability to express TH *in vivo* and may even do so at low but undetectable levels. Such potentiality for TH expression, however, is transient and may be held in check by intrinsic inhibitory molecules, possibly originating from target structures *in vivo*. According to this proposition, when cortical cells are explanted to culture before receiving this inhibitory influence (E14) they are able to

express the enzyme. At later times in development, after cells innervate target structures, their phenotypic potentiality becomes restricted.

We are not at all certain which neurons in the mature cortex correspond to those capable of expressing TH in the embryo. However, since the TH-containing neurons withdraw from the cell cycle before day 13 of gestation, in advance of the majority of developing cortical cells (Duckett and Pearse, 1968; Raedler and Sievers, 1976; Konig et al., 1977; Raedler and Raedler, 1978), the most interesting possibility is a group of neurons first discovered by Ramon y Cajal (1891) and later described by Retzius (1893). These so-called Cajal-Retzius cells are the first neurons generated in the mammalian cortex, beginning on E11 (Duckett and Pearse, 1968; Raedler and Sievers, 1976; Konig et al., 1977; Raedler and Raedler, 1978). In addition to the timing of their terminal mitosis, these cells are morphologically heterogeneous (Raedler and Raedler, 1978), similar to the TH-containing cortical neurons. Taken together, these findings raise the possibility that the cortical neurons which are competent to express TH may comprise at least a subpopulation of Cajal-Retzius cells in the cortex. Alternatively, these neurons may correspond to a recently described population of perikarya that transiently express TH from postnatal days 14 to 30 in the rat neocortex (Berger et al., 1985), or to some as yet undefined population of cells.

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