Effects of a Novel Differentiation Factor on the Development of Catecholamine Traits in Noncatecholamine Neurons from Various Regions of the Rat Brain: Studies in Tissue Culture

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The muscle-derived differentiation factor called MDF initiated expression of the catecholamine (CA) enzyme tyrosine hydroxylase (TH) in non-CA neurons isolated from a variety of regions in the rat brain. Specifically, subpopulations of neurons from the striatum, collicular plate, and cerebellum were TH-immunoreactive after an overnight exposure to MDF in culture. The number of immunopositive cells was greatest in the striatum, where more than half of all plated neurons expressed the enzyme. In contrast, MDF had no effect on the central neurons of the hippocampus or on peripheral sensory neurons. In ³H-thymidine studies, only brain neurons that had already withdrawn from mitosis expressed TH. These cells remained open to the epigenetic influence of MDF only during a brief and defined critical period that appears to be timed intrinsically. Without daily replenishment of MDF, expression of the enzyme disappeared after several days in culture, suggesting that MDF was rapidly depleted or degraded *in vitro*. However, in the continued presence of MDF, TH expression was maintained indefinitely, thus producing a permanent alteration in phenotype. Moreover, a single exposure to MDF during the critical period was sufficient to render neurons permanently receptive to the molecule so that TH expression could be reinitiated many days later. It is postulated that a memory of this biochemical interaction was established in these neurons, making transmitter phenotypic plasticity possible at later stages.

During the developmental process known as differentiation, neurons acquire the cell-specific traits that together constitute their phenotype. One of the critical phenotypic decisions neurons make is the selection of which neurotransmitters they will use at their synapses. Regrettably, our understanding of the molecular steps that lead to this decision remains inadequate. However, over a decade of work on the PNS has provided valuable insight into certain aspects of the process. In these studies on rat sympathetic neurons, environmentally derived substances have been shown to play a key role in directing the final choice of neurotransmitter (Bunge et al., 1978; Le Douarin, 1980; Yamamori et al., 1989; Schotzinger and Landis, 1990).

Thus, sympathetic neurons, which normally express only catecholamine-associated traits, also display cholinergic phenotypic characters when grown in the presence of certain agents (Johnson et al., 1976; Patterson and Chun, 1977; Iacovitti et al., 1981; Kessler, 1985). Conversely, cholinergic neurons of the parasympathetic nervous system express adrenergic traits if exposed to the appropriate environmental cues *in vitro* (Iacovitti et al., 1985; Teitelman et al., 1985) and *in vivo* (Coulombe and Bronner-Fraser et al., 1986; Landis et al., 1987). These agents have been called differentiation factors because of their ability to influence phenotypic decisions without affecting cell survival.

To date, several putative differentiation factors with activities in the PNS have been described (Sah and Matsumoto, 1987; Adler et al., 1989; Yamamori et al., 1989). Because of their extraordinarily low concentration in tissues, isolation and characterization of the pure molecules have been extremely difficult. Recently, however, the cholinergic neuronal differentiation factor has been purified to homogeneity (Weber, 1981; Fukada, 1985) and a cDNA encoding it has been sequenced (Yamamori et al., 1989). As described previously, this protein, which is secreted by cultured rat heart cells, promotes expression of cholinergic traits in sympathetic neurons (Patterson and Chun, 1977). Interestingly, in addition to neurons, this molecule also influences the differentiation of myeloid and other cell types, suggesting that some differentiation factors may play multiple roles in development.

While it has long been anticipated that such factors also regulate the differentiation of brain neurons, a candidate substance has only recently been proposed. This putative molecule, called muscle-derived differentiation factor or MDF, has profound effects on the expression of transmitter-specific genes in cultured brain neurons (Iacovitti et al., 1989). As its name implies, MDF has been extracted and partially purified from rat muscle cells (L6 cell line). In culture, it has the unique capacity to initiate transcription of the gene for tyrosine hydroxylase (TH), the first and limiting enzyme in catecholamine (CA) biosynthesis, in cells where the gene is normally quiescent. Without change in neuronal survival, MDF increases by 10-fold the levels of mRNA-encoding TH and its corresponding protein in cultured cells from the embryonic rat cerebral cortex (Iacovitti et al., 1989). While many known substances can amplify the levels of TH protein in CA neurons (for review, see Zigmond et al., 1989), this is the first example of a factor that initiates expression of a CA gene in non-CA neurons from brain.

As is the case for many known growth factors (Perez-Polo, 1987), MDF exerts its influence on the genome only at a very precise stage in the development of the neuron. Thus, the ce-

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rebral cortex of rats at 13 d gestation are dramatically affected by MDF; however, by 16 d gestation, cortical neurons are no longer responsive to the molecule (Iacovitti et al., 1987). The existence of a critical period for the actions of MDF raises questions about the regulation of this timed event and the role that it plays in influencing the initial choice of neurotransmitter and the potential for transmitter plasticity at later stages.

This study sought to answer some of the fundamental questions raised by our earlier studies on the biological actions of MDF, including whether other neurons from the CNS as well as the PNS respond to MDF and, if so, whether the response is universally the same. Experiments were designed to investigate the timing of the critical period for the effects of MDF and the regulatory site for this event. Finally, the long-term consequences of MDF treatment on the phenotypic fate of neurons were studied. The findings of the present study demonstrate that the effects of MDF are confined to the CNS, and there, only to specific subpopulations of neurons. As in the cortex, neurons from elsewhere in the brain are responsive to MDF only during a critical period that appears to be intrinsically timed in each cell type. As well as promoting the initial appearance of TH, MDF perpetuated expression of the enzyme when it was continually present in the culture. This resulted in a permanent alteration in neurotransmitter phenotype. Moreover, a single exposure to MDF during the operative period renders neurons MDF-responsive at later times. It is suggested that this early developmental "experience" establishes a biochemical memory of induction in the cell and thereby provides a mechanism for phenotypic plasticity at later stages in life.

Materials and Methods

Tissue culture. Pregnant Sprague-Dawley rats were purchased from Taconic Lab Animals at 12 d gestation $\pm$ 12 hr fertilization day = embryonic day (E) 0. On E13, E14, E16, or E18, rats were anesthetized with pentobarbital, and the embryos were removed. After dissection of the brain from the cranium, the cerebral cortices, striatum, collicular plate, hippocampus, and cerebellum were isolated. After removal of the meninges, tissue was incubated in Ca^{2+} -, Mg^{2+} -free Hanks' balanced salt solution (CMF-HBSS) for 10 min at 37°C in a clinical rotator (40 rpm). The incubation mixture was replaced with a 0.01% trypsin solution (in CMF-HBSS) and incubated for another 15 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). Peripheral dorsal root ganglia (DRG) isolated from embryos between E16 and E19 were stripped of adherent connective tissue and incubated in CMF-HBSS for 10 min at 37°C before being transferred to a 0.25% trypsin solution for 30 min. Tissue was rinsed in L15 and then placed in media as described above with the addition of 100 ng/ml purified nerve growth factor (kind gift of Dr. E. M. Johnson, Washington University Medical School, St. Louis, MO). Cells were dissociated by trituration through a reduced-bore glass pipette and plated onto glass Lab Tek culture dishes coated either with polymerized collagen or with polyornithine (precoated for 24 hr at room temperature at a concentration of 0.1 mg/ml in 15 mM borate buffer, pH 8.4). The cellular plating density was approximately 5×10^5 cells/dish. Striatal cells formed fewer cell aggregates on polyornithine than on collagen; thus, in radioautography experiments, which required that all cells be evenly coated with emulsion, cells were grown on polyornithine. In all other experimental manipulations, collagen was used.

Preparation of partially purified MDF. To prepare soluble extract of muscle, cultures of nearly confluent L6 muscle cells were grown for 10 d *in vitro*, harvested, homogenized in 0.01 M sodium phosphate buffer (pH 7.2, 60×10^6 cells/ml) for 1–2 min with a polytron, and centrifuged at $40,000 \times g$ for 1 hr to separate soluble and insoluble fractions. The clear supernatants were collected, the protein levels were determined, and the supernatants were treated with acid. By lowering the pH to 2 with HCl for 1 min on ice and then raising it back to pH 6 with NaOH,

90% of the protein was precipitated with no loss of MDF activity. This fraction was then passed over a reverse-phase C18 column and MDF was recovered in the flow through fraction. This step removed a yellow chromophore (probably carnitine, which is concentrated in muscle) that is toxic to the cells in culture. This fraction was then passed over a DEAE-cellulose anion exchange column (Whatman DE-52). MDF was weakly bound under these conditions, and 60% of the activity could be recovered after elution from the column. This $\approx 10,000$ -fold-purified molecule(s) was concentrated by lyophilization with 90% recovery and resuspended in culture media (pH 7.8) for use in experiments.

Immunocytochemistry. Cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and processed with antibodies to TH (1:5000 dilution, kind gift of Dr. T. H. Joh, Cornell University Medical College, New York, NY) using the immunoperoxidase/avidin-biotin complex (ABC) method of staining (Elite Vectakit). Except where stated otherwise, TH⁺ cell numbers were determined by counting stained cells in all microscopic fields on the culture dish. This was accomplished with the aid of an eyepiece reticule used at a $10\times$ magnification.

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

Results

Effects of MDF on TH expression in various regions of the nervous system. Our earlier studies demonstrated that, in culture, the soluble muscle-derived factor, designated MDF, induces synthesis of TH and its encoding mRNA in 10% of cultured neurons from the embryonic rat cerebral cortex (Iacovitti et al., 1989). The present study sought to determine whether MDF affected other non-CA neurons of central and also peripheral origin. In these experiments, cultures of dissociated cells were established from various regions of the brain and PNS (see Materials and Methods). All cultures were stabilized on a complete media overnight and subsequently transferred into either control media or media supplemented with partially purified MDF. After 2 d *in vitro*, cultures were fixed and processed for the localization of TH antibodies.

As was previously observed in the cerebral cortex, cultures generated from other non-CA regions of the CNS, including striatum, collicular plate, and cerebellum, contained low percentages of TH-immunoreactive neurons even when grown in control media (Table 1). However, the number of TH neurons in these cultures was markedly increased by growing the cells in media containing MDF. The greatest increment was found in the striatum (52-fold) (Table 1), with approximately 50% of the neurons expressing TH after an overnight exposure to MDF (Fig. 1). Smaller increases were observed in the colliculus (fourfold) and cerebellum (fivefold) (Table 1). Interestingly, however, not all brain regions were MDF-responsive; cultures of hippocampal neurons were devoid of TH immunoreactive neurons under control conditions, and they did not express TH after MDF supplementation at any of the ages tested (Table 1).

In contrast to the dramatic actions of MDF on CNS neurons, no measurable effect was observed in sensory neurons of the PNS. Consequently, the number of TH-immunopositive neurons of the dorsal root ganglia was not increased by MDF treatment (Table 1).

These results suggest that specific populations of neurons from some but not all brain regions respond to MDF with enhanced TH expression, as evidenced by the unexpected appearance of TH immunoreactivity in cells. In contrast, non-CA sensory neu-

Figure 1. Effect of MDF on TH expression in cultures of E14 caudate nucleus. Photomicrographs are shown of striatal neurons grown either on control media (*A*) or in the presence of MDF (*B*) for 1 d before being processed for the immunocytochemical localization of TH.

rons of the PNS do not express TH in response to MDF treatment in culture.

Effects of MDF on the critical period. Our earlier studies demonstrated that cerebral cortical neurons had the ability to respond to cues in culture and express TH only during a brief critical period in their embryogenesis (Iacovitti et al., 1987). To assess whether other MDF-responsive neurons shared this property of stage dependence, cultures were generated from a variety of brain regions isolated from embryos ranging in age from E14 to E20. As was found previously, neurons from other brain regions were MDF-competent only during restricted periods in their development (Fig. 2). This period occurred between E14 and E16 in collicular plate and cerebellar cultures. However, in striatal cultures, the critical period was protracted, extending at

least up to the time of birth. The number of MDF-responsive neurons, however, steadily declined during this period.

For each brain region, the critical period appeared to correspond to the time just following peak withdrawal of neurons from the mitotic cycle (DeLong and Sidman, 1962; Altman, 1963; Berry et al., 1964; Fentress et al., 1981). This notion was tested by incubating neurons of varying age in media containing ^3H -thymidine. After an 18-hr exposure, cultures were fixed and processed for simultaneous localization of TH by immunocytochemistry and ^3H -thymidine incorporation by radioautography. Although the majority of unstained cells were labeled autoradiographically, no TH-immunoreactive neurons from any regions tested contained silver grains over their nuclei (data shown only for striatal neurons in Fig. 3). The absence of label

Table 1. Comparison of MDF-induced changes in TH expression in neurons from the CNS and PNS

	MDF treatment	Number of TH ⁺ neurons/culture	Fold induction
CNS			
Cerebral cortex	–	1222 ± 118	12
	+	14,631 ± 991	
Striatum	–	431 ± 78	52
	+	22,542 ± 769	
Collicular plate	–	461 ± 93	4
	+	1,909 ± 440	
Cerebellum	–	129 ± 28	5
	+	658 ± 104	
Hippocampus	–	0	0
	+	0	
PNS			
Dorsal root ganglion	–	2	0
	+	3	

Embryonic rats were dissected at varying developmental ages. Brain cells from cerebral cortex, striatum, cerebellum, collicular plate, and hippocampus (E13–20) and peripheral neurons from dorsal root ganglia (E16–19) were dissociated and plated in culture. All cultures were stabilized on a complete media overnight and subsequently transferred into either control media or media supplemented with partially purified MDF. After incubation for 2 d *in vitro*, cultures were fixed and processed for the localization of TH antibodies and the number of TH-immunoreactive neurons was counted in all microscopic fields. Values represent means ± SEM of triplicate cultures from two separate platings.

was not due to the dilution of ³H that occurs after successive divisions since the length of the cell cycle is 11 hr (von Waechter and Jaensch, 1973). Instead, the absence of radioisotope incorporation indicates that these cells have not synthesized DNA and thus probably are not replicating while in culture. The extended critical period observed in striatum presumably reflects the fact that striatal neurons continue to be generated, though to a lesser extent, even into the first week of postnatal life (Fentress et al., 1981).

Regulation of the timing of the critical period. The fact that neurons remain responsive to MDF for only a brief critical period after withdrawing from mitosis raises the question as to whether the timing of this event is intrinsically preprogrammed in the cell or occurs as the result of some critical interaction with other developing cells. To distinguish between these possibilities, cortical cells from E13 rat were placed in culture, thus isolating them from cellular interactions *in vivo*. Neurons were allowed to “age,” presumably beyond the critical period, by growing them in culture for 7 d on control media. Cultures were

Table 2. Timing of critical period for MDF induction of TH is set by internal clock

Age of donor cortex	Days <i>in vitro</i> prior to MDF treatment	Number of TH ⁺ neurons/culture
E13	1	38,946 ± 4662
E13	7	11 ± 3

The cerebral cortex was isolated from rats at 13 d of gestation, dissociated, and grown in culture on control media. After 1 or 7 d in culture, media was replaced with MDF-supplemented feed. One day later, cultures were fixed and stained with TH antibodies, and the number of TH neurons/culture was determined. Values represent means ± SEM of duplicate cultures from two separate platings.

Figure 2. Cultures of dissociated striatum, collicular plate, and cerebellum were established from embryos at a variety of different developmental stages. After 1 d *in vitro*, cultures were supplemented overnight with MDF before TH immunocytochemical processing. Values are expressed as a percentage of maximal induction (number of TH⁺ neurons) for each brain region. Maximal values are provided in Table 1.

then supplemented with MDF from culture day 7 to 9 before immunocytochemical analysis. Cultures treated in this manner were devoid of TH-positive neurons (Table 2), suggesting that the timing of the critical period may be internally regulated.

Effects of MDF on the fate of TH expression. Even when responsive neurons are exposed to MDF during their critical period, the induced expression of TH is short-lived, disappearing after only a few days *in vitro* (Iacovitti et al., 1987). To test whether MDF, in addition to initiating expression of TH, might also prolong the presence of the enzyme there, cerebral cortical cells were plated in culture either on media containing MDF or on control media. After 2 d, several cultures from each group were fixed and processed for immunocytochemistry. All remaining cultures were maintained on control media until day 8 when they were fixed and stained, and the number of TH neurons was determined. As expected, after 2 d *in vitro*, MDF-treated cultures contained eightfold more TH neurons than did control cultures (Fig. 4). However, expression of the enzyme in MDF-supplemented cultures, as in controls, rapidly declined. By 7 d *in vitro*, only an occasional TH-positive neuron was present. To determine whether the loss of TH expression resulted from the depletion and/or degradation of MDF with increasing incubation time, some cultures of cortical cells were replenished daily with MDF-supplemented media. In these cultures, TH expression was maintained at 7 d *in vitro* (Fig. 4) and

Figure 3. Combined radioautography of ^3H -thymidine and TH immunocytochemistry in cultures of E14 striatum. Note that cells containing TH immunoreactivity do not contain silver grains over their nuclei.

at later times (14 d in culture; data not shown). These results suggest that MDF can both initiate and perpetuate TH expression indefinitely in cultured cortex but that the factor is rapidly depleted or degraded with time *in vitro*.

Finally, in this series of experiments, the possibility was examined that a single exposure to MDF during the critical period was sufficient to produce long-lasting effects on TH. To test this, cultures of E13 cortical cells were grown in MDF-containing media during the 2 d immediately following plating and then replaced with control media for the next 10 d before receiving a final overnight exposure to MDF. Cultures were then processed for immunocytochemical analysis. As demonstrated in Table 3, untreated neurons or neurons treated after close of the critical period were not affected by MDF. However, a single exposure to MDF during the critical period rendered cortical neurons permanently receptive, such that TH expression could be reinduced by MDF as much as 14 d later.

Discussion

It has previously been reported that the muscle-derived substance called MDF promotes the differentiation of CA traits in cultured neurons from the rat cerebral cortex (Iacovitti et al., 1989). The present study demonstrates that MDF produces a

Figure 4. Cultures of dissociated E13 rat cerebral cortical cells were established in control media or media supplemented with MDF. After the initial treatment with MDF, supplemented cultures were split into two groups: one was fed daily with control media, the other with MDF-supplemented media. Cultures were fixed after 2 or 7 d *in vitro*, and then the number of TH-immunoreactive neurons was determined. Numbers represent means $\pm$ SEM of triplicate cultures from two separate platings.

similar response in neurons from a wide variety of neural regions. Assessed in this study were areas from the CNS and PNS composed, nearly in their entirety, of neurons synthesizing neurotransmitters other than CAs. After overnight incubation with MDF, subsets of neurons from most but not all brain regions tested expressed TH, the first enzyme in the CA biosynthetic pathway. The proportion of responsive cells varied from one region to another, with the most dramatic effect occurring in cultured striatal neurons. These responses were limited to neurons of the CNS; MDF had no observable effect on peripheral neurons from the sensory ganglia.

Table 3. Phenotypic changes in TH expression after single exposure of MDF in cortical cultures

Culture number	MDF treatment (d)				Number of TH ⁺ neurons/culture
	0	1	2–12	13	
1	–	–	–	–	96 $\pm$ 12
2	–	+	+	+	8649 $\pm$ 752
3	–	+	–	–	111 $\pm$ 29
4	–	–	–	+	116 $\pm$ 16
5	–	+	–	+	6468 $\pm$ 412

Cultures of E13 cerebral cortex were supplemented with MDF-containing media at various times in culture. After 14 d, cultures were fixed and stained, and the number of TH⁺ neurons was determined. Values represent means $\pm$ SEM of duplicate cultures from two platings.

As in the cerebral cortex (Iacovitti et al., 1987), other non-CA neurons were responsive to MDF only during well-delineated stages in their embryonic development. In all regions, this period corresponded to the time just following peak cell proliferation (DeLong and Sidman, 1962; Altman, 1963; Berry et al., 1964; Fentress et al., 1981). Thus, only neurons that had recently withdrawn from the mitotic cycle were susceptible to the effects of MDF in culture; neurons isolated from rats several days later in embryogenesis did not respond. This finding raised questions as to whether the timing of this event was genetically preprogrammed in the neuron or occurred as the result of some critical interaction with other developing cells. Indeed, during the early postmitotic period, many important cell–cell interactions are established *in vivo*, including contact with target and innervating neural structures. It is possible that a critical exchange with one or more of these cells fixed the phenotype of the neuron *in vivo* and, in so doing, precluded its ability to respond to MDF in culture. Arguing against this interpretation was the finding that neurons plated soon after their terminal mitosis and aged several days in culture before MDF treatment did not respond to treatment. Thus, similar to their *in vivo* counterparts, cultured cortical neurons developed a critical period for MDF without benefit of external influences. The findings of this study are, therefore, more consistent with the hypothesis that neurons contain an internal clock that regulates the timing of their responsiveness to MDF. However, because of the complexity of cell types in our cultures, we cannot eliminate the possibility that the critical period is dependent upon cellular interactions that are not removed by the cell culture process.

Even when competent neurons were treated with MDF during the critical period, TH expression was short-lived, disappearing after several days in culture. However, if replenished daily, MDF perpetuated the presence of the enzyme indefinitely, suggesting that (1) MDF was either rapidly depleted or degraded *in vitro*; (2) MDF was capable not only of initiating expression of TH but also of maintaining it; and (3) MDF could, in this way, effect a permanent change in neuronal phenotype.

Quite remarkably, a single exposure to the factor at the appropriate developmental stage allowed cortical neurons to reexpress TH after a second addition of MDF as much as 14 d later. As expected, neurons that did not receive this initial treatment remained unaffected by a later MDF challenge. These findings suggest that a “biochemical memory” of the effects of MDF was established in MDF-primed neurons. Priming of the neurons rendered them permanently receptive to the actions of the molecule and thus provided a mechanism for reexpression of the enzyme at later stages of life. It is possible that early interactions of the genome with differentiation factors are requisite steps for achieving phenotypic plasticity in the adult.

The findings of this study have addressed the issues of where in the nervous system MDF might be acting, when the molecule is most apt to be effective, and, in part, how MDF achieves its results. Less clear from these studies is why MDF so profoundly impacts certain neurons and not others. Do MDF-responsive neurons from these diverse locales in the brain share any one feature in common? One possibility under consideration is that responding neurons belong to a particular neurotransmitter class. Although direct proof is lacking, there is mounting circumstantial evidence to implicate the neurotransmitter GABA. Most compelling is the fact that over 90% of intrinsic neurons in the striatum are GABAergic in the adult rat (Mugnaini and Oertel, 1985). This fact, taken together with the finding in culture that

more than half of the nondividing neurons of the striatum express TH after MDF treatment, strongly suggests that TH-containing striatal neurons are (or will be) GABAergic. Further suggestive of a possible relationship between TH and GABA in these neurons are the results of preliminary double-labeling experiments on the cerebral cortex (L. Iacovitti, unpublished data). In that study, it was shown that 5 d after MDF treatment, when the proportion of TH-positive neurons was declining and that of GABA-positive neurons was rising, occasional cells colabeled for both antigens. This suggested that TH expression was being downregulated in the same cortical neurons in which GABA was appearing. Experiments are currently in progress to substantiate these results further and to determine whether the coexpression of TH and GABA can be extended if cultures are maintained on MDF-supplemented media.

Interestingly, all regions that responded to MDF with an increase in the number of TH neurons always contained some TH-positive cells even under control culture conditions (in the absence of the factor). Conceivably, the appearance of TH in control cells might be due to the liberation of MDF or other differentiation factors during the dissociation procedure. Alternatively, this population may represent those occasional TH cells previously noted *in vivo*. Indeed, TH-immunoreactive neurons have been observed in all MDF-responsive brain regions, including the cerebral cortex (Gaspar et al., 1987; Kosaka et al., 1987a), striatum (Berger et al., 1985; Kosaka et al., 1987b; Satoh and Suzuki, 1990), and inferior colliculus (Jaeger and Joh, 1983). At least in the case of the cerebral cortex (Kosaka et al., 1987a), these cells also express GABA (Kosaka et al., 1987b). It is conceivable that these TH/GABA cells are vestiges of an earlier developmental stage. According to this hypothesis, certain neurons in the brain are capable of expressing genes related to both the CA and GABA transmitter systems if exposed to appropriate differentiation signals at the correct times in development. In most cases, TH expression is downregulated, possibly as a result of the disappearance of CA-inducing factors, while GABA expression is upregulated. However, in some instances, coexpression is maintained even in the adult brain (Kosaka et al., 1987b). Proof of the existence of cells with this shared lineage awaits further investigation using double-labeling techniques.

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