

TYROSINE HYDROXYLASE ACTIVITY DECREASES WITH INDUCTION OF CHOLINERGIC PROPERTIES IN CULTURED SYMPATHETIC NEURONS¹

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Received December 13, 1982; Revised February 25, 1983; Accepted March 1, 1983

Abstract

Establishment of transmitter phenotype is an essential step in neuronal development. Studies on rat sympathetic neurons both *in vivo* and *in vitro* have provided evidence that mature cholinergic sympathetic neurons arise from previously noradrenergic neurons. Cultured rat superior cervical ganglion neurons can be influenced by their environment to remain noradrenergic, to acquire dual transmitter function, or to become predominantly cholinergic. Several other neuronal traits, such as a variety of surface molecules and released proteins, change simultaneously with levels of catecholamine and acetylcholine production, suggesting that various components of transmitter phenotype are regulated in concert. In this report, tyrosine hydroxylase levels are compared in neurons cultured under noradrenergic, dual function, or cholinergic conditions. Both enzyme activity in cell extracts and immunocytochemical staining were measured. These methods showed significantly less tyrosine hydroxylase enzyme activity and immunoreactive material in cholinergic cultures compared to noradrenergic and dual function cultures. These data support the interpretation that a switch in transmitter status from noradrenergic to cholinergic has occurred. This interpretation contrasts with that of Iacovitti et al. (Iacovitti, L., T. H. Joh, D. H. Park, and R. P. Bunge (1981) *J. Neurosci.* 1: 685-690), who conducted their experiments under critically different culture conditions.

The transmitter phenotype of a neuron is comprised of numerous differentiated properties, including the enzymes necessary for transmitter synthesis and breakdown, the vesicles for storage, and in some cases, the membrane sites for reuptake. One interesting question concerning control of gene expression is whether the various elements of a phenotype are controlled coordinately during development. For example, some cells of the sympathoadrenal lineage, adrenal chromaffin cells, acquire three characteristic enzymes of the catecholamine (CA) biosynthetic pathway at approximately the same time in embryogenesis (Cochard et al., 1978, 1979; Teitelman et al., 1979). However, in other cell types of this lineage, only one or two of the enzymes may develop. Neuronal differentiation can involve not only acquisition

of particular elements of a phenotype, but their loss as well. For example, some autonomic noradrenergic neurons change their transmitter phenotype during ontogenesis. In developing sweat glands of the rat footpad, the noradrenergic properties of the early innervation are gradually reduced, as the innervation becomes cholinergic. As these axons become positive for the acetylcholinesterase cytochemical reaction, endogenous CA stores are lost and immunocytochemical staining for tyrosine hydroxylase (TH) and dopamine β -hydroxylase become greatly reduced (Landis and Keefe, 1980, 1983b; Siegel et al. 1982). Although the mature cholinergic axons lose their endogenous CA stores, they maintain to some extent the distinctive noradrenergic capability to take up exogenous CA.

These findings on the differential loss of adrenergic properties with attainment of cholinergic development *in vivo* are in qualitative agreement with earlier studies on the cholinergic differentiation of sympathetic neurons *in vitro*. Varying the culture environment of neonatal rat superior cervical ganglion neurons can produce a spectrum of transmitter types ranging from nearly purely adrenergic to dual function, to predominantly cholinergic neurons (Reichardt and Patterson, 1977; Potter et al., 1981). Under culture conditions favoring expression of

¹ This work was supported by grants from the National Institute of Neurological and Communicative Disorders and Stroke. E. W. is a predoctoral trainee of the National Institute of General Medical Sciences and P. H. P. is a Rita Allen Foundation Scholar and a McKnight Foundation Neuroscience Development Awardee. We wish to thank Doreen McDowell, Geraldine Spencer, Allison Doupe, and Shirley Wilson for assistance with the cell cultures and with the preparation of the manuscript. We also thank Tom Jessell for advice on photography. Antiserum against tyrosine hydroxylase was a gift from J. Thibeault.

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cholinergic functions, development of certain noradrenergic properties is inhibited. These include accumulation of endogenous CA stores (Landis, 1976, 1980; Johnson et al., 1976, 1980), the ability to synthesize and store CA (Patterson et al., 1975; Patterson and Chun 1977a, b; Schaffner and Daniels, 1982) and the presence of an isozyme of monoamine oxidase associated with noradrenergic neurons (Pintar et al., 1981). However, such cholinergic neurons still retain the ability to take up exogenous CA via the high affinity uptake I system (Reichardt and Patterson 1977; Wakshull et al., 1978; Landis, 1980), as do the mature cholinergic sympathetic axons of the rat footpad. The degree of suppression of development of noradrenergic properties is dependent on the length of time and the concentration of conditioned medium factor applied (Johnson et al., 1976; Patterson and Chun, 1977a, b; Landis, 1980). When neurons are only weakly cholinergically induced, individual cells retain the ability to secrete both transmitters indefinitely (Furshpan et al., 1976; Potter et al., 1981).

Recent reports have confirmed and extended the previous description of the dual function state. Cultured neurons which have formed cholinergic synapses can exhibit immunocytochemical staining for TH (Higgins et al., 1981) and TH and choline acetyltransferase enzyme activities can increase in parallel in culture (Iacovitti et al., 1981). However, these authors hypothesize that the inhibition of development of CA synthesis and accumulation and the loss of CA stores in neurons grown under strongly cholinergic conditions are not caused by failure of CA synthetic enzymes to develop, but rather are caused by acetylcholine (ACh) competing with CA for storage within the same vesicle population (Johnson and Higgins, 1981). This model predicts that CA synthesis, but not storage, would increase steadily with time in culture in parallel with ACh production. In the present report, however, it is shown that there are significant differences in both TH specific activity and TH immunocytochemical staining in neurons grown under strongly cholinergic versus adrenergic conditions, that this difference depends on the strength of the cholinergic induction, and that the difference in TH activity parallels earlier data on CA synthesis and accumulation by living neurons.

Materials and Methods

Cell culture. Neuronal cultures were prepared essentially by the method of Hawrot and Patterson (1979), except that cell dissociation was accomplished enzymatically rather than mechanically. Superior cervical ganglia of newborn rats were dissected, cleaned of extraneous tissue, and then incubated at 37°C for 1 to 2 hr in a solution of proteolytic enzymes. This enzyme solution consisted of 5 mg/ml of dispase (neutral protease from *Bacillus polymyxa* Grade II, Boehringer-Mannheim) and 1 mg/ml of collagenase (126 units/mg; Worthington) in Ca²⁺, Mg²⁺-free Hanks' balanced salt solution supplemented with glucose, antibiotics, and glutamine, as described for plating medium (Hawrot and Patterson, 1979). For each 10 ganglia, 1 ml of enzyme solution was used. Dissociation to single cells was effected by 4 to 6 passes through an 18 gauge, 1-inch syringe needle. The resulting suspension was washed three times by low

speed centrifugation in plating medium (without NaHCO₃) to remove the dispase and collagenase and then was restored to an appropriate volume with plating medium. Since large numbers of non-neuronal cells are released during enzymatic dissociation, the suspension was plated directly into growth medium containing 10⁻⁵ M cytosine-1-β-D-arabinofuranoside (Sigma Chemical Co.) A 1-week exposure to this antimetabolite virtually eliminated non-neuronal cells from the cultures. Growth medium consisted of carbonate-buffered L15 (Flow Labs) supplemented with glucose, antibiotics, glutamine, and fresh vitamins (Hawrot and Patterson, 1979), 1 μg/ml of nerve growth factor prepared by the method of Bocchini and Angeletti (1969), and 5% adult rat serum. Depolarizing growth medium for the production of noradrenergic cultures contained 20 mM KCl exchanged for an equal amount of NaCl. Cultures were used for experiments between 3 and 4 weeks *in vitro*.

Heart cell-conditioned medium. Serum-free heart cell-conditioned medium was prepared according to the method of Fukada (1980) and was partially purified and concentrated 10-fold by ammonium sulfate precipitation as described by Weber (1981). The concentrate was mixed with equal parts of growth medium containing a double supplement of glucose, glutamine, antibiotics, fresh vitamins, nerve growth factor, and rat serum.

TH assay. TH was assayed by the method of Hendry and Iversen (1971) with modifications. Approximately 3000 neurons were homogenized on ice in ground glass microhomogenizers (200 μl capacity) in 0.8 M sodium phosphate buffer at pH 6 with 0.05% Triton X-100. A total volume of 10 μl of neuronal extract and extraction buffer was added to an equal volume of assay cocktail. The final concentrations of reagents in this mixture were: 0.0015% brocrescine, an inhibitor of DOPA decarboxylase (gift of American Cyanamid Co.), 0.15 M β-mercaptoethanol (Sigma), 1.4 nM 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride (Calbiochem), 25 μM L-tyrosine (Sigma), 1 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, and 0.025% Triton X-100. The reaction was initiated by addition of 5 μl of pretreated, tritium-labeled tyrosine. L-[ring-2,6-³H]tyrosine (New England Nuclear (NEN)) of specific activity 30 Ci/mmol at a concentration of 1 mCi/ml in 0.01 N HCl was neutralized with 0.1 N NaOH and incubated with 2 to 3 mg of alumina (WN3; Sigma) at room temperature for 1 hr. The reaction was linear for up to at least 10 μl of neuronal extract for 15 min. Assays were routinely terminated after 10 min by addition of ice-cold 0.4 N perchloroacetic acid containing 8 μg/ml of L-DOPA (Sigma). Alumina pencil columns were used to collect [³H]DOPA as described (Hendry and Iversen, 1971). Protein was measured in aliquots of neuronal extract by the bromosulphophthalein binding method of Wallace and Partlow (1978), using bovine serum albumin as a standard.

Metabolic labeling of neurotransmitters. Norepinephrine, dopamine, and ACh production were measured in living cells by scintillation counting of the electrophoretically separated metabolites of L-[ring 2,6-³H]tyrosine (30 Ci/mmol; NEN) and [methyl-³H]choline chloride (80 Ci/mmol; NEN). Isotopic incubations and paper electrophoresis were carried out as described previously (Mains and Patterson, 1973).

Immunocytochemical staining. Cultures were fixed with 4% paraformaldehyde in 0.12 M phosphate buffer for 40 min at room temperature and rinsed three times with phosphate-buffered saline. They were then incubated for 30 min at 37°C with a rabbit antiserum generated against TH purified from rat pheochromocytoma (Thibeault et al., 1981), diluted 1:3000 in a high ionic strength buffer consisting of 0.5 M NaCl in 10 mM phosphate buffer at pH 7.4 which contained 10% horse serum, 0.3% Triton X-100, and 0.1% sodium azide. The diluted antibody was incubated on ice for 1 hr before use with horse serum. Primary antibody binding was visualized by the method of Sternberger (1979), using peroxidase-antiperoxidase (Boehringer-Mannheim) with a Hanker-Yates chromagen and cobalt intensification. The second antibody was sheep anti-rabbit serum. To facilitate comparisons, photographs were taken at the same exposure and printed under identical conditions.

Results

Three different culture conditions were used to control transmitter choice. The most cholinergic cultures were obtained by supplementing the medium with partially purified cholinergic inducing factor from serum-free medium conditioned by neonatal rat heart cells (Fukada, 1980). The resulting neuronal growth medium was concentrated 5-fold for the conditioned medium factor (CM) compared to the original heart culture supernatants. On the other hand, the neurons can be maintained in their original noradrenergic state, and they are resistant to the effects of the cholinergic factor if they are depolarized by electrical stimulation, veratridine treatment, or elevation of external K^+ concentration (Walicke et al., 1977). The elevated K^+ method was used here. Cultures of intermediate transmitter status were grown in nondepolarizing medium and without CM.

Transmitter synthesis in living neurons was determined by metabolic labeling with [3H]tyrosine and [3H]choline. In Figure 1, *a* and *b*, are shown the content of

metabolically labeled ACh and CA accumulated per neuron under each culture condition. As previously reported, the production of these transmitters is reciprocally related under conditions of strong cholinergic induction (Patterson and Chun, 1977a). CA production in cultures grown with CM is only one-fifth that of the most noradrenergic cultures, whereas ACh production is increased 20-fold. In the intermediate condition examined here, ACh and CA are produced in similar amounts.

TH activity was assayed in homogenates of sister cultures of those used for metabolic labeling. The specific activity of TH in extracts from cells grown under each culture condition in a representative experiment is shown in Figure 1*c*. TH activity is similar in extracts from cultures grown under noradrenergic conditions and from cultures of intermediate transmitter status. Activity in extracts from cholinergic cultures is about 5-fold lower. Thus, measurement of TH activity in extracts parallels that of CA synthesis and accumulation by metabolic labeling. This correspondence between the two measures of CA production has been observed in six separate platings. TH specific activity in extracts of cultures of intermediate transmitter status is comparable to that reported by Iacovitti et al. (1981) and Swerts et al. (1983) for dual function cultures.

To determine whether the cholinergic culture extract contained a TH-inhibitory activity, a mixing experiment was performed. Equal amounts of extracts from cultures grown under adrenergic and cholinergic conditions were combined and assayed. The TH specific activity of this mixture was the average of the activities of the two contributing extracts (data not shown). No inhibitory activity of the cholinergic extract or enhancing activity of the noradrenergic extract was detected.

The presence of TH in cultures was also assessed by immunocytochemical staining using the peroxidase-antiperoxidase technique. Intense staining of neuronal somas and light staining of neuronal processes were seen in cultures grown under depolarizing conditions (Fig. 2*a*).

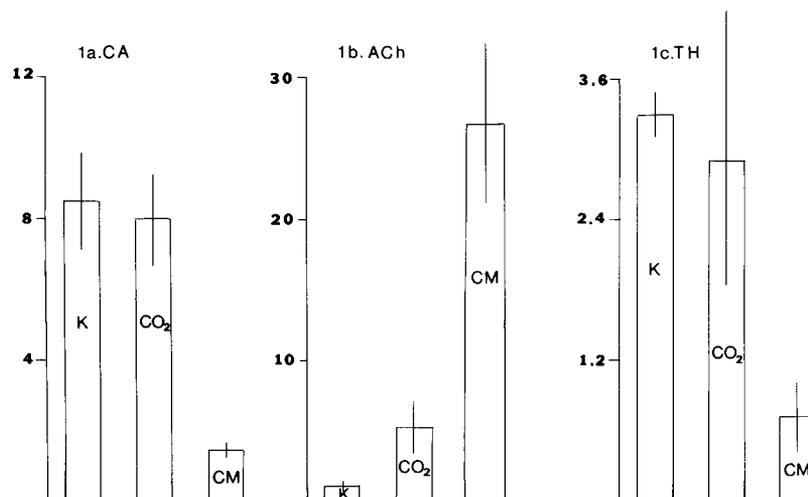


Figure 1. CA and ACh production, and TH activity in cultured sympathetic neurons. *a*, CA synthesis measured by metabolic labeling as described under "Materials and Methods," in femtomoles per neuron (mean \pm SD for three cultures). *b*, ACh synthesis measured by metabolic labeling as described in the text, in femtomoles per neuron (mean \pm SD for three cultures). *c*, TH activity in culture extracts assayed as described under "Materials and Methods," expressed as picomoles of DOPA per μ g of protein per 10 min (mean and range of values for two different extracts, each containing three pooled cultures). *K*, cultures grown in high potassium; *CO*, cultures grown without depolarization or heart cell conditioned medium; *CM*, cultures grown with heart cell conditioned medium.

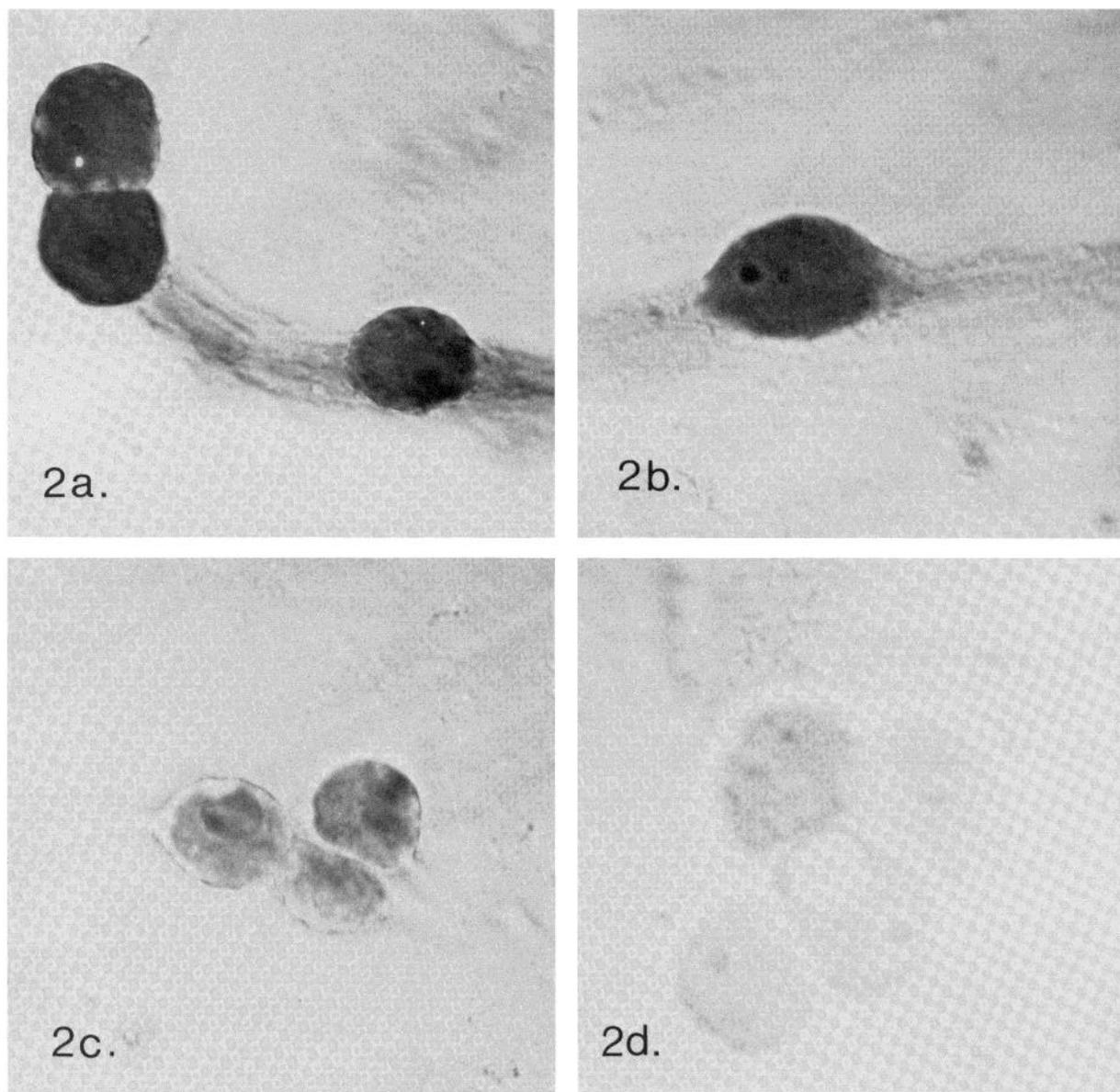


Figure 2. TH staining of cultured sympathetic neurons. *a*, Cells were grown in high potassium. *b*, Cells were grown without depolarization or heart cell conditioned medium. *c*, Cells were grown with heart cell conditioned medium. *d*, Cells were grown without depolarization or heart cell conditioned medium and were processed without primary antibody.

Somas of dual function neurons stained somewhat less darkly, and process staining was also less evident (Fig. 2*b*). Processes of neurons grown in CM were unstained, but their somas, though much lighter than those of the noradrenergic and dual function cultures, were significantly darker than the background obtained in the absence of primary antibody (Fig. 2, *c* and *d*).

Discussion

The data reported here show that TH activity in extracts of cultured sympathetic neurons correlates well with levels of CA synthesis and accumulation measured by metabolic labeling of living cells. Furthermore, TH activity and immunocytochemical staining is inversely related to levels of ACh production in cultures subjected to a strong cholinergic induction. Metabolic labeling

measures the combined results of transmitter synthesis, storage, release, reuptake, and degradation. Theoretically, a change in any one of these processes would change the net production of CA. It is also possible that rates of synthesis and degradation could change together in a compensatory manner, leaving final CA production levels unchanged. The observation that the activity of the rate-limiting enzyme of CA synthesis, TH, parallels the metabolic labeling of CA suggests that this is the case, and that the processes affecting breakdown of CA are regulated in concert rather than separately. This is consistent with the linkage of the expression of other noradrenergic indicators such as endogenous CA stores (Johnson et al., 1976; Landis, 1980), the presence of the adrenergic form of monoamine oxidase (Pintar et al., 1981), certain spontaneously released (Sweadner, 1981) and surface membrane glycoproteins (Braun et al., 1981)

and glycolipids (Zurn, 1983) enriched in noradrenergic neurons, and increases in certain lectin- (Schwab and Landis, 1981) and monoclonal antibody-binding sites (Chun et al., 1980). These markers of the noradrenergic state are good candidates for coordinate regulation. These noradrenergic traits are regulated reciprocally with cholinergic functions, but this is only apparent when a strong cholinergic induction is achieved. Thus it is possible that Iacovitti et al. (1981) did not observe early leveling off of TH activity because their cultures were not exposed to sufficient levels of cholinergic inducing factor.

Even in predominantly cholinergic cultures, decreased but still detectable amounts of CA are produced (Fig. 1b). Low levels of TH activity can also be detected in extracts of these cultures (Fig. 1c), and faint immunocytochemical staining for TH can be observed in fixed cultures (Fig. 2). The most straightforward interpretation of these findings is that TH is present in cholinergically induced neurons, but in lower amounts than in cells grown under noradrenergic conditions. The possibility that the amount of enzyme protein is the same in both types of extracts, but that its activity is modulated by an inhibitor or an activator, was tested by assaying mixed homogenates. No such effect was detected. The observation that the immunocytochemical staining results agree qualitatively with the activity data also argues against this possibility.

The failure of TH activity and immunocytochemical staining to fall to zero in even the most cholinergic cultures parallels the observation of faint immunocytochemical staining for TH and dopamine β -hydroxylase in mature cholinergic sympathetic fibers of the rat sweat gland (Siegel et al., 1982). In addition, the ability to take up exogenous CA persists after cholinergic development both *in vivo* (Landis and Keefe, 1980, 1983a) and *in vitro* (Landis, 1976; Reichardt and Patterson, 1977; Wakshull et al., 1978). The significance of these reduced but detectable noradrenergic characteristics in mature cholinergic sympathetic neurons is unknown. The ontogeny of sympathetic neurons involves a migration of neural crest cells along pathways which appear to exert an adrenergic influence on neuronal precursors (Le Douarin, 1980). Later in development, the small percentage of the population which becomes cholinergic does so by undergoing a phenotypic transition. This mechanism for producing cholinergic sympathetic neurons may result in traces of CA biosynthetic enzymes and uptake sites being present as vestiges of their developmental history. It is possible that an even stronger cholinergic induction would result in the complete disappearance of these traits, but complete obliteration of the noradrenergic phenotype is not necessary *in vivo* and is not yet attained *in vitro*. Another possibility is that complete loss of these markers does not occur because these functions are a necessary part of the final phenotype.

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