Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3526–3530, June 1983
Neurobiology

Expression of catecholaminergic characteristics by primary sensory neurons in the normal adult rat *in vivo*

(phenotypic expression/neurotransmitter phenotype/vagal afferents/tyrosine hydroxylase/neuronal plasticity)

DAVID M. KATZ*, KEITH A. MARKEY*, MENEK GOLDSTEIN†, AND IRA B. BLACK*

*Division of Developmental Neurology, Cornell University Medical College, Department of Neurology, 515 East 71st Street, New York, New York 10021; and †Neurochemistry Laboratory, Department of Psychiatry, New York University Medical Center, 550 1st Avenue, New York, New York 10016

Communicated by Michael Heidelberger, March 14, 1983

ABSTRACT **Expression of catecholaminergic characteristics by primary sensory neurons was examined in the vagal nodose and glossopharyngeal petrosal ganglia of the normal adult rat *in vivo*. Catecholaminergic phenotypic expression was documented by immunocytochemical localization of tyrosine hydroxylase (TyrOHase; EC 1.14.16.2), radiochemical assay of specific TyrOHase catalytic activity, and cytochemical localization of formaldehyde-induced catecholamine fluorescence (FIF) within principal ganglion cells. The TyrOHase-containing cells exhibited morphologic features typical of primary sensory neurons, such as an initial axon glomerulus and a single, bifurcating neurite process. These cells were distinguished from TyrOHase- and FIF-positive small intensely fluorescent cells by size, morphology, and staining intensity. TyrOHase-containing neurons appeared to be insensitive to neonatal treatment with 6-hydroxydopamine, thereby distinguishing them from sympathetic neurons. Nodose and petrosal ganglia of adult rats exhibited TyrOHase catalytic activity, linear with respect to tissue concentration over a 10-fold range, indicating that the immunoreactive enzyme was functional. Transection of specific ganglionic nerve roots depleted TyrOHase catalytic activity and neuronal immunoreactivity within the petrosal ganglion, suggesting that target organ innervation regulates enzyme levels within ganglion perikarya. Our study indicates that primary sensory neurons express catecholaminergic transmitter traits in the normal adult rat. Consequently, in the periphery, catecholaminergic characters are not restricted to the sympathoadrenal axis but are expressed by functionally and embryologically diverse populations of autonomic neurons.**

Recent studies of neurotransmitter function in peripheral systems have begun to define molecular mechanisms regulating neuronal phenotypic expression. Autonomic neurons, for example, are capable of expressing multiple transmitter phenotypes; the apparent choice of transmitter depends, at least in part, on cues in the cellular microenvironment. In dissociated cell culture, noradrenergic sympathetic neurons express cholinergic properties in the presence of nonneuronal cells or conditioned medium (1, 2). Moreover, sympathetic neurons can express the putative peptide transmitter, substance P, *in vivo* and *in vitro* (3, 4). Extensive evidence suggests, however, that substance P is also a *sensory* transmitter (5). Consequently, these studies not only document remarkable transmitter plasticity but also suggest that expression of common transmitter characters is shared by *functionally* diverse neurons.

To address this issue further, we have been examining catecholaminergic expression in peripheral neurons that differ functionally and embryologically. In the periphery, catecholaminergic phenotypic characters have classically been associated with neural crest derivatives, including sympathetic neurons, small intensely fluorescent (SIF) cells, and adrenomedullary cells. The present study was undertaken to determine whether catecholaminergic characters are also normally expressed by other classes of peripheral neurons. We examined expression of catecholamine biosynthetic enzymes and formaldehyde-induced catecholamine fluorescence (FIF) in primary sensory neurons in the nodose ganglion (NG) and petrosal ganglion (PG) of the adult rat. The enzymes were tyrosine hydroxylase (TyrOHase; tyrosine 3-monooxygenase, EC 1.14.16.2), which catalzyes the rate-limiting step in catecholamine biosynthesis (6), and dopamine β-hydroxylase (DBOHase; dopamine β-monooxygenase, EC 1.14.17.1) and phenylethanolamine *N*-methyltransferase (PNMTase, EC 2.1.1.28), which catalyze the synthesis of norepinephrine and epinephrine, respectively.

The NG and PG contain the cells of origin of vagal and glossopharyngeal afferent fibers, respectively. Avian studies demonstrated that NG and PG neurons are derived from the embryonic epibranchial placodes (7). These neurons were selected for the present study, therefore, because they differ functionally and embryologically from sympathetic neurons. Our study was performed in *adults*, because (transient) catecholaminergic expression had already been demonstrated in presumptive neuroblasts of the embryo *in vivo* (8–10). Consequently, we sought to determine whether heterogeneous populations of neurons express catecholaminergic characters during maturity as well as development *in vivo*.

MATERIALS AND METHODS

Animals. Three- to 6-week-old female Sprague–Dawley rats from Hilltop Lab Animals (Scottdale, PA) were housed as described (11).

Staining Procedures. Animals were perfused by the technique of Berod *et al.* (12) with minor modifications. Tissues were placed in 30% sucrose (0.1 M sodium phosphate buffer, pH 7.4) overnight at 4°C and cut at 10 μm on a cryostat and sections were melted onto gelatin-coated glass slides. Slides were heated to 55°C for 15 min and rinsed for 15 min in phosphate buffer (0.1 M, pH 7.4).

Immunofluorescence. Sections were processed by previously described techniques (8) with minor modifications. Primary antisera were rabbit anti-rat TyrOHase (13) or rabbit preimmune serum, 1:200 in Tris·HCl buffer, 0.05 M, pH 8.6; guinea pig anti-rat DBOHase, 1:250; or goat anti-bovine PNMTase, 1:200, in 0.15 M NaCl/0.1 M sodium phosphate, pH 7.40, containing 0.3% Triton X-100 (Fisher). Secondary antisera were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TyrOHase, tyrosine hydroxylase; DBOHase, dopamine β-hydroxylase; PNMTase, phenylethanolamine *N*-methyltransferase; NG, nodose ganglion; PG, petrosal ganglion; SIF cells, small intensely fluorescent cells; FIF, formaldehyde-induced catecholamine fluorescence; 6-OH-dopamine, 6-hydroxydopamine.

fluorescein isothiocyanate-conjugated goat anti-rabbit, rabbit anti-goat, or goat anti-guinea pig IgG (GIBCO) diluted 1:100 in Tris·HCl buffer (0.05 M, pH 8.6) with 0.3% Triton X-100.

Peroxidase–anti-peroxidase staining. Slides were treated by published techniques (14) with minor modifications to eliminate endogenous peroxidase activity. The slides were then soaked in 1% sheep serum, 0.05 M Tris·HCl, pH 7.6/0.15 M NaCl (Tris/saline) for 30 min to reduce nonspecific staining. Sections were air dried, covered with primary antibody (rabbit anti-rat TyrOHase) as defined above, and incubated at room temperature for 1 hr. Slides were then rinsed in two changes of Tris/saline and air dried, and sections were incubated at room temperature for 30 min with a pool of secondary antibody (sheep anti-rabbit IgG), 1:100 in Tris/saline with 1% sheep serum. The slides were next rinsed in two changes of Tris/saline for 10 min, covered with rabbit peroxidase–anti-peroxidase (Miles), 1:100, with Tris/saline, 1% sheep serum, and incubated at room temperature for 30 min. After two changes of Tris/saline and air drying, sections were covered with 0.05% 3,3′-diaminobenzidine·4HCl (Sigma) and 0.01% H_2O_2 in 0.1 M phosphate buffer for 5–20 min at room temperature. After several rinses in distilled water, slides were transferred to 0.1% osmium tetroxide in distilled water for 1 min, rinsed several times in distilled water, dehydrated in graded alcohol solutions, cleared in xylene, and coverslipped with Permount (Fisher).

FIF. Tissues were processed by published techniques (8) with minor modifications.

TyrOHase Catalytic Assay. Tissue samples containing the PG and the rostral one-third of the NG (normally fused *in situ*) were assayed as described (15).

Surgical Procedures. While the rats were under halothane anesthesia the glossopharyngeal nerve was transected proximal to the origin of the carotid sinus nerve. The carotid sinus nerve and other glossopharyngeal nerve branches were transected at their origin from the main nerve trunk.

Drug Treatment. Neonatal rats were injected with 6-hydroxydopamine (6-OH-dopamine) at 50, 100, 150, or 200 mg/kg, intraperitoneally, on days 2, 4, 6, and 8 of life. The drug was dissolved in 0.15 M NaCl containing ascorbic acid at 0.2 mg/ml. Injection volumes ranged between 0.10 and 0.20 ml. Littermate controls received equal-volume injections of vehicle only.

RESULTS

Immunocytochemistry. To determine whether or not the NG or PG contain catecholaminergic biosynthetic enzymes, tissue sections were incubated with specific antisera to TyrOHase, DBOHase, or PNMTase. Immunocytochemical staining revealed TyrOHase immunoreactive neurons and nerve fibers in the NG and PG of 3- and 6-week-old rats. No positively stained structures were seen in tissue sections incubated with preimmune serum (Fig. 1). No DBOHase- or PNMTase-immunoreactive neurons were observed in either of the ganglia. However, control sections of adrenal medulla, processed simultaneously, contained intense DBOHase and PNMTase immunoreactivity, indicating the efficacy of the staining procedure.

Many of the TyrOHase-stained neurons exhibited morphologic features typical of primary sensory neurons, including an initial axon glomerulus (Fig. 2*A*) and a single, bifurcating neuritic process (Fig. 2*B*). TyrOHase-positive neurons in the PG made up a considerably larger proportion of the total ganglion cell population than did the positively stained cells in the NG.

TyrOHase immunoreactivity was also localized to cells resembling SIF cells. These cells, which were often clustered in groups close to blood vessels, differed markedly in size, mor-

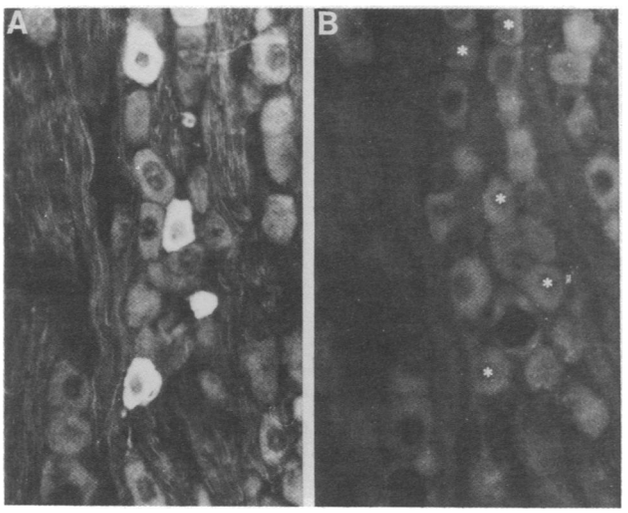

FIG. 1. Fluorescence photomicrographs of adjacent serial sections through the NG stained with rabbit anti-rat TyrOHase antibody (*A*) and rabbit preimmune serum (*B*). Asterisks in *B* indicate the location of cells that also appear in *A* and are TyrOHase immunoreactive. (×270.)

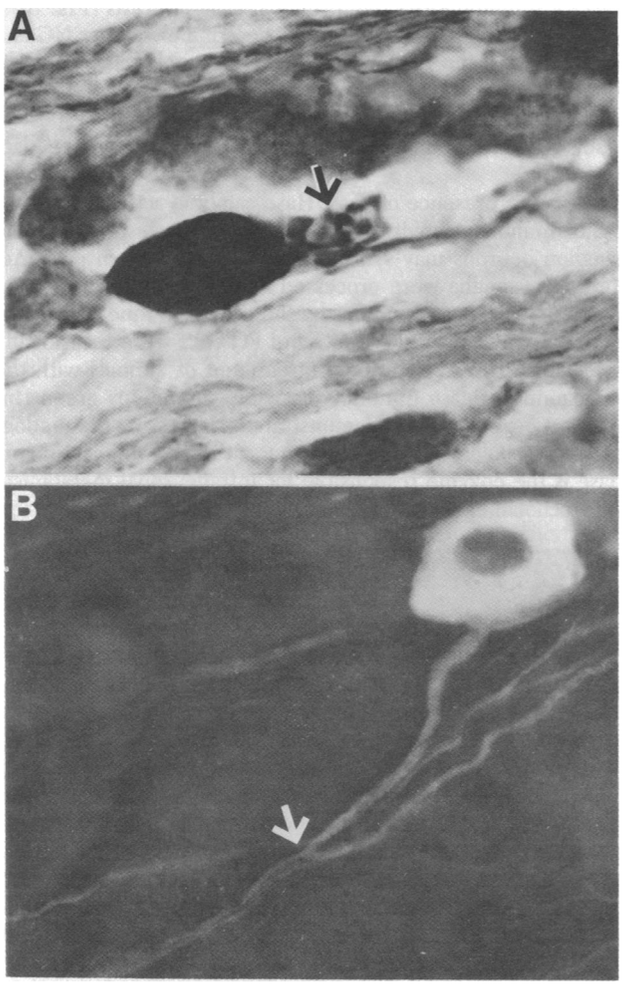

FIG. 2. (*A*) Bright-field photomicrograph of a TyrOHase-immunoreactive NG cell, showing the initial axon glomerulus (arrow). (Peroxidase–anti-peroxidase stained preparation; ×440.) (*B*) Fluorescence photomicrograph of a TyrOHase-immunoreactive NG cell, showing the bifurcating neuritic process (arrow). (Fluorescein isothiocyanate-stained preparation; ×440.)

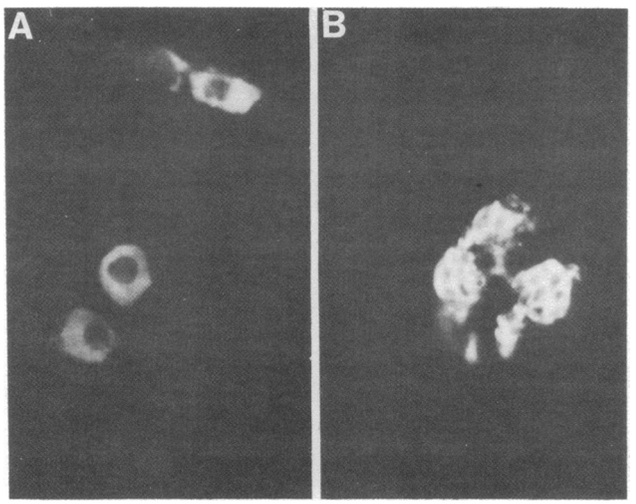

FIG. 3. Fluorescence photomicrographs of TyrOHase-stained NG neurons (*A*) and SIF cell-like cells (*B*) from the same ganglion. (×280.)

phology, and staining characteristics from the TyrOHase-positive neurons. The SIF cell-like cells were small, had scant cytoplasm, and were much more intensely stained than the TyrOHase-immunoreactive principal ganglion cells (Fig. 3).

TyrOHase Catalytic Activity. To determine whether NG and PG cells contain *functional* TyrOHase molecules, specific TyrOHase catalytic activity was measured. Abundant catalytic activity was, in fact, detectable and was linear with respect to tissue concentration over an approximately 10-fold range (data not shown).

FIF. The presence of TyrOHase catalytic activity and TyrOHase immunoreactivity in the NG and PG suggested that some ganglion neurons may synthesize catecholamines *in vivo*. Accordingly, ganglia were processed for the histochemical demonstration of biogenic amines by the FIF technique. In normal animals, faint FIF-positive NG and PG cells were occasionally detectable. However, after pretreatment of animals with the monoamine oxidase inhibitor pargyline, numerous green fluorescent perikarya were demonstrated, suggesting that endogenous catecholamine synthesis occurs (Fig. 4). Monoamine oxidase is the enzyme responsible for intraneuronal metabolism of catecholamines (16).

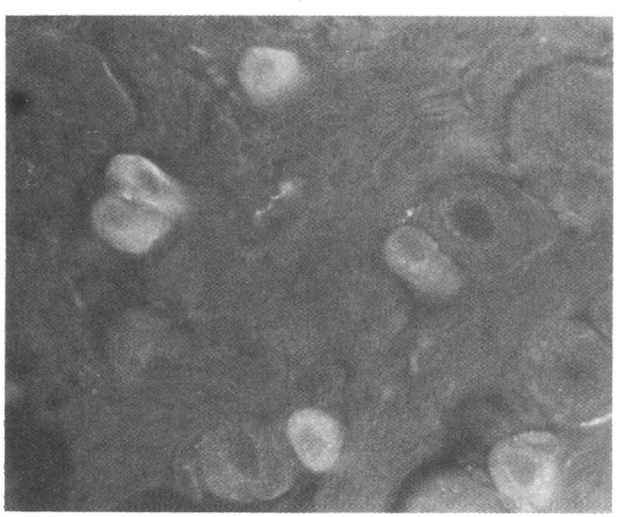

FIG. 4. Fluorescence photomicrograph of FIF in adult NG neurons after treatment of the animal with pargyline, a specific inhibitor of monoamine oxidase. (×410.)

Effects of Sympathetic Destruction. Treatment of neonatal rats with 6-OH-dopamine selectively destroys sympathetic catecholaminergic neurons throughout the animal (17). To determine whether catecholaminergic NG and PG neurons are similarly affected, we treated neonates with 6-OH-dopamine and examined the ganglia after maturation. Injection of the neurotoxin had no discernible effect on the number or staining intensity of TyrOHase-immunoreactive or FIF-positive neurons in the NG and PG. In striking contrast, the adjacent sympathetic superior cervical ganglion, which served as a control, exhibited a profound decrease in neuron number, indicating the efficacy of treatment. Furthermore, the number of FIF-stained *fibers* passing through the NG and PG was markedly reduced.

Effects of Axotomy. In sympathetic ganglia, postganglionic axotomy or inhibition of axonal transport reduces TyrOHase activity (11), suggesting that target innervation plays a regulatory role. To determine whether TyrOHase in sensory neurons is similarly regulated, we examined the effects of peripheral axotomy on activity in the PG. The glossopharyngeal nerve (nerve IX) and its branches contain the peripheral processes of PG neurons. To examine the effects of axotomy, the carotid sinus nerve, a branch of nerve IX containing PG fibers that innervate the carotid body and sinus, was unilaterally transected. In each animal the contralateral intact PG and carotid sinus nerve served as a control. TyrOHase activity in the PG decreased by more than 50% one week after the operation (Fig. 5). Activity remained significantly depressed for at least 3 weeks after surgery (Fig. 5).

To determine whether decreased catalytic activity was accompanied by altered immunoreactivity, ganglia were examined immunocytochemically after surgery. Nerve transection profoundly decreased the apparent number and staining intensity of TyrOHase-immunoreactive perikarya. The effect was most pronounced after transection of the entire nerve IX proximal to the origin of the carotid sinus nerve (Fig. 6). Transec-

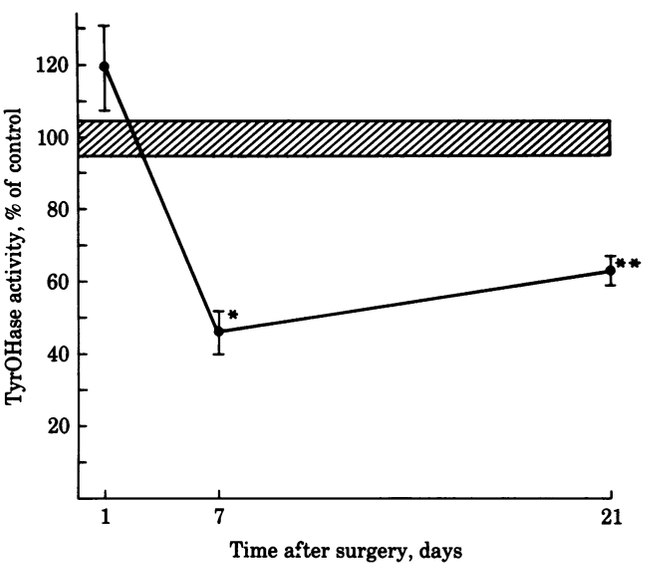

FIG. 5. Effect of carotid sinus nerve transection on TyrOHase activity within the PG. Surgery was performed on the left side and the unoperated right ganglion served as control. Each value represents the mean ± SEM for the operated side expressed as a percentage of the contralateral control value. The hatched area represents the standard error of the grand mean of all control values. TyrOHase activity in control ganglia averaged 4.4 pmol/hr per ganglion. The 1-day value does not differ significantly from its control.
* Differs from control, $P < 0.001$.
** Differs from control, $P < 0.05$.

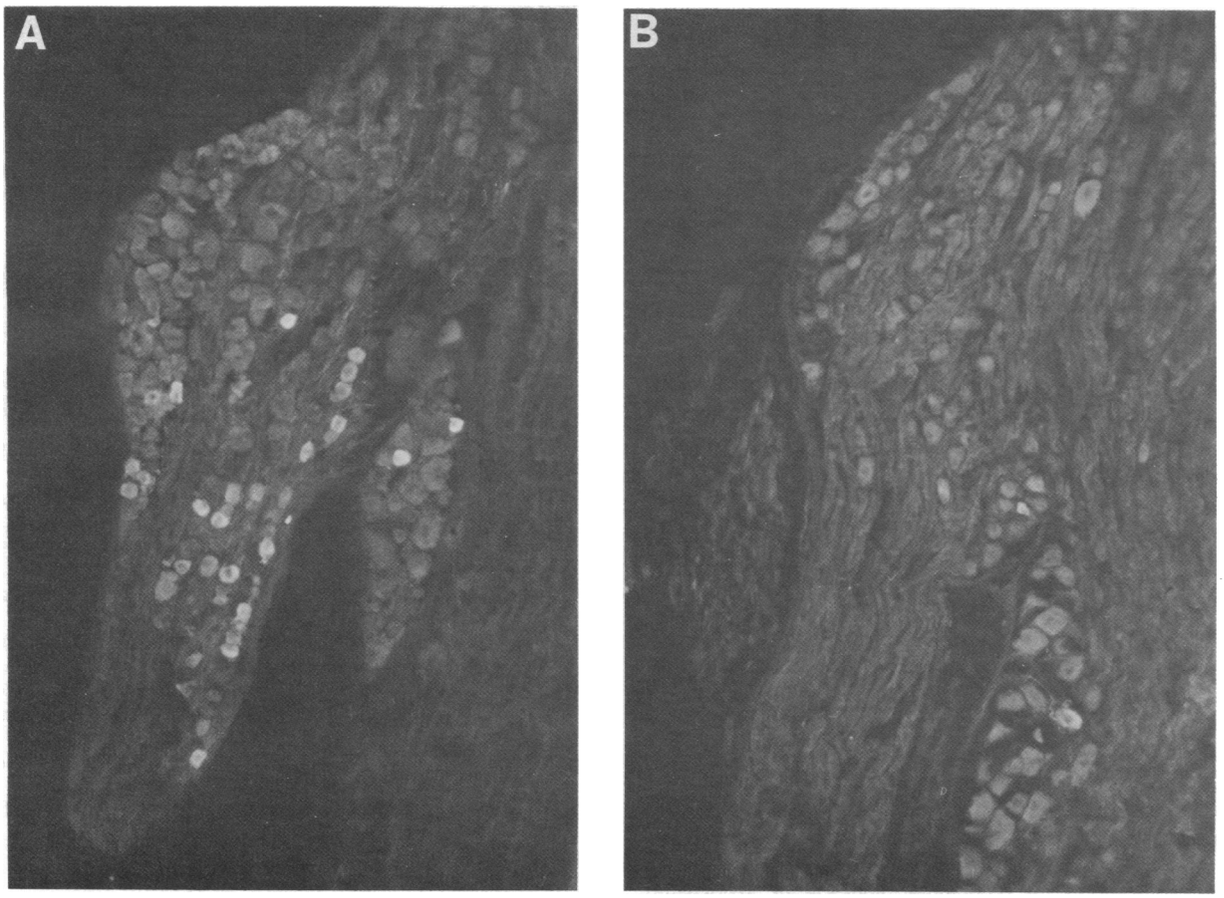

FIG. 6. Fluorescence photomicrographs of TyrOHase-stained PG sections contralateral (*A*) and ipsilateral (*B*) to carotid sinus nerve transection, one week after surgery. (Fluorescein isothiocyanate-stained preparations; ×100.)

tion distal to the carotid sinus nerve had no effect on TyrOHase staining.

DISCUSSION

Our studies indicate that NG and PG neurons in the adult rat normally express catecholaminergic transmitter characters *in vivo*. Consequently, catecholamines are not restricted to the sympathoadrenal axis in the periphery but may subserve autonomic *sensory* functions as well.

Cellular Identity. Several lines of evidence indicate that these ganglion cells are primary sensory neurons, in contradistinction to other potential catecholaminergic elements, such as SIF cells (18, 19) or ectopic sympathetic neurons (20). The TyrOHase-positive ganglion cells exhibit specific morphologic characteristics typical of primary sensory neurons. The initial axon glomerulus is a prominent feature of principal neurons in mammalian sensory ganglia (21, 22) that is not shared by any other class of neurons, including sympathetic neurons or SIF cells (20, 21). Similarly, the presence of a single bifurcating neurite is typical, though not diagnostic, of the sensory phenotype. [For example, parasympathetic neurons may also give rise to a single, branched process (23); however, such cells are not localized to the NG or PG (20, 24)].

The PG and NG catecholaminergic cells differ markedly in size, shape, and staining intensity from SIF cells (Fig. 3), further differentiating them from these elements. Finally, the PG and NG neurons are apparently impervious to the destructive effects of neonatal 6-OH-dopamine, distinguishing these neurons from sympathetic neurons pharmacologically (see below). Consequently, morphologic and pharmacologic criteria indicate that these ganglion cells are primary sensory neurons, and not SIF cells or ectopic sympathetic neurons.

Nature of the Phenotypic Characters Expressed. Abundant evidence suggests that the TyrOHase immunoreactivity exhibited by NG and PG neurons represents authentic enzyme. We used a highly specific and well-characterized antiserum, raised against purified rat pheochromocytoma TyrOHase (13). Previous studies demonstrated no crossreactivity of the antiserum with any other molecules (13).

The present studies, moreover, indicate that the immunoreactive TyrOHase in NG and PG neurons is functional: independent radiochemical assay demonstrated abundant TyrOHase catalytic activity in the ganglia. Further, TyrOHase catalytic activity and cellular immunoreactivity exhibited parallel responses to postganglionic axotomy, suggesting that catalytic activity faithfully reflected intraneuronal immunoreactivity (see below).

Whereas the NG and PG neurons expressed abundant TyrOHase immunoreactivity, DBOHase and PNMTase, which synthesize norepinephrine and epinephrine, were not detectable. This did not simply represent intrinsic insensitivity of the methods, because (*i*) control tissues stained positively and (*ii*) our previous work has demonstrated these antigens at low levels during development (25). TyrOHase therefore seems to be expressed independently of DBOHase and PNMTase in these neurons, consistent with the contention that the different catecholaminergic biosynthetic enzymes are differentially expressed in neuronal populations (26).

The expression of TyrOHase, but not DBOHase or PNMTase, further implies that these neuronal populations may synthesize

dopamine, but not norepinephrine or epinephrine. The presence of FIF in the PG and NG is consistent with this possibility. Moreover, this observation supports the contention that TyrOHase is functionally active within the ganglia *in vivo*. Preliminary double staining experiments for TyrOHase and FIF indicate that the same neurons express TyrOHase and catecholamine fluorescence.

The marked enhancement of FIF by inhibition of monoamine oxidase suggests that PG and NG neurons also express the enzymes that normally catabolize intraneuronal catecholamines. This indirect evidence suggests that sensory neurons, as well as sympathetic and central catecholaminergic neurons, express functional monoamine oxidase.

Finally, the lack of destruction of PG and NG neurons by neonatal 6-OH-dopamine treatment may also provide information regarding phenotypic expression. The neurotoxin is specific for catecholaminergic neurons, because it is selectively taken up and concentrated by the stereospecific, energy-requiring, high-affinity uptake sites localized to catecholaminergic neuronal membrane (27, 28). Consequently, absence of a response in PG and NG neurons implies, but does not prove, that these neurons do not express the uptake system. The dissociation of expression of high-affinity uptake and synthetic enzymes has been previously reported for developing catecholaminergic populations (9, 29). The present studies provide additional evidence that these characters may be differentially expressed (26).

Regulation of TyrOHase. In sympathetic neurons, catecholaminergic phenotypic characters are regulated by a variety of environmental factors, including neuronal activity and neuron–target interactions (30). In the superior cervical ganglion, for example, transection, or colchicine blockade of axoplasmic transport decreases TyrOHase catalytic activity (11). Consequently target organ innervation plays a critical role in the regulation of sympathetic TyrOHase. To determine whether similar mechanisms regulate TyrOHase in sensory neurons, we performed peripheral nerve transection, separating the PG from its carotid body and carotid sinus targets. Peripheral axotomy significantly reduced TyrOHase catalytic activity and simultaneously depleted TyrOHase immunoreactivity within PG neurons. While these decreases may be attributable to a number of mechanisms, these observations raise the possibility that targets regulate transmitter phenotypic characters in PG neurons, paralleling target regulation in other populations (30).

In summary, NG and PG neurons, sensory derivatives of embryonic epibranchial placodes, normally express catecholaminergic phenotypic characters in the adult *in vivo*. Consequently, transmitter characters traditionally thought to be restricted to the sympathoadrenal axis in the periphery, are actually expressed by neurons that differ embryologically, anatomically, and functionally. Nevertheless, common mechanisms, such as target regulation, may govern the activity and levels of phenotypic characters in these diverse populations.

The authors gratefully acknowledge the expert assistance of Mrs. Dana Straka, Ms. Teresa Larraz, Ms. Elise Grossman, and Mrs. Bettye Mayer. This work was supported by a National Institutes of Health Postdoctoral Fellowship (NS 06623-02) to D.M.K. and National Institutes of Health Grants NS 10359, HD 12108, and NS 06801 and was aided by a grant from the Dysautonomia Foundation, Inc.

1. Patterson, P. H. (1978) *Annu. Rev. Neurosci.* **1**, 1–17.
2. Bunge, R., Johnson, M. & Ross, C. D. (1978) *Science* **199**, 1409–1415.
3. Kessler, J. A., Adler, J. E., Bohn, M. C. & Black, I. B. (1981) *Science* **214**, 335–336.
4. Kessler, J. A. & Black, I. B. (1982) *Brain Res.* **234**, 182–187.
5. Otsuka, M. & Konishi, S. (1976) *Cold Spring Habor Symp. Quant. Biol.* **40**, 135–143.
6. Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) *J. Pharmacol. Exp. Ther.* **148**, 1–8.
7. LeDouarin, N. M., Smith, J. & LeLievre, C. S. (1981) *Annu. Rev. Physiol.* **43**, 653–671.
8. Cochard, P., Goldstein, M. & Black, I. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2986–2990.
9. Jonakait, G. M., Wolf, J., Cochard, P., Goldstein, M. & Black, I. B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4683–4686.
10. Teitelman, G., Baker, H., Joh, T. H. & Reis, D. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 509–513.
11. Kessler, J. A. & Black, I. B. (1979) *Brain Res.* **171**, 415–424.
12. Berod, A., Hartman, B. K. & Pujol, J. F. (1981) *J. Histochem. Cytochem.* **29**, 844–850.
13. Markey, K. A., Kondo, S. & Goldstein, M. (1980) *Mol. Pharmacol.* **17**, 79–85.
14. Fisher, M., Gapp, D. A. & Kozak, T. (1981) *Dev. Brain Res.* **1**, 341–354.
15. Black, I. B. (1975) *Brain Res.* **95**, 170–176.
16. Kopin, I. J. (1964) *Pharmacol. Rev.* **16**, 179–191.
17. Angeletti, P. U. & Levi-Montalcini, R. (1970) *Proc. Natl. Acad. Sci. USA* **65**, 114–121.
18. Grillo, M. A., Jacobs, C. & Comroe, J. H. (1974) *J. Comp. Neurol.* **153**, 1–14.
19. Kojima, H., Svetake, K., Yokoo, H., Anraku, S., Inanaga, K., Higashi, H., Nishi, S., Yamamoto, T. & Ochi, J. (1981) *Experientia* **37**, 1332–1333.
20. Lieberman, A. R. (1976) in *The Peripheral Nerve*, ed. Landon, D. N. (Wiley, New York), pp. 188–278.
21. Ramon y Cajal, S. (1909) *Histologie du Systeme Nerveux* (Consejo Superia de Investigaciones Cientificas, Madrid, Spain), Vol. 1.
22. Katz, D. M. & Karten, H. J. (1980) *J. Comp. Neurol.* **193**, 549–564.
23. Purves, D. & Hume, R. (1981) *J. Neurosci.* **1**, 441–452.
24. Stensaas, L. & Fidone, S. J. (1977) *Brain Res.* **124**, 29–39.
25. Bohn, M. C., Goldstein, M. & Black, I. B. (1981) *Dev. Biol.* **82**, 1–10.
26. Black, I. B. (1982) *Science* **215**, 1198–1204.
27. Thoenen, H. & Tranzer, J. P. (1968) *Arch. Pharmak. Exptl. Pathol.* **261**, 271.
28. Jonsson, G. & Sachs, C. (1970) *Eur. J. Pharmacol.* **9**, 141–155.
29. Landis, S. C. & Keefe, D. (1980) *Soc. Neurosci. Abstr.* **6**, 379.
30. Black, I. B. (1978) *Annu. Rev. Neurosci.* **1**, 183–214.