0270-6474/85/0506-1633$02.00/0

The Journal of Neuroscience
Vol. 5, No. 6, pp. 1633–1643
June 1985

Appearance of the Noradrenergic Markers Tyrosine Hydroxylase and Neuropeptide Y in Cholinergic Nerves of the Iris Following Sympathectomy[1]

HÅKAN BJÖRKLUND,[2] TOMAS HÖKFELT, MENEK GOLDSTEIN,* LARS TERENIUS‡ AND LARS OLSON

*Department of Histology, Karolinska Institute, S-104-01 Stockholm, Sweden, *Department of Psychiatry, New York University Medical Center. New York, New York 10016, and ‡Department of Pharmacology, Uppsala University, 75123 Uppsala, Sweden*

Abstract

Selective autonomic denervations of the iris have been used to study the possible redistribution of adrenergic markers within adult nerve fiber systems and to reveal the cellular origin of a nonsympathetic fiber plexus induced to express such markers.

The presence and distribution of fibers showing neuropeptide Y (NPY)- and tyrosine hydroxylase (TH)-like immunoreactivity was studied in the rat iris using stretch-prepared whole mounts. Normal irides contained a dense regular network of NPY-positive varicose fibers. Such fibers were regularly seen innervating blood vessels. The choroid membrane had a high number of fluorescent fibers. A similar, although slightly denser TH-positive fiber system was visualized in the iris. One or 2 days after surgical removal of the superior cervical ganglion, almost all NPY- and TH-positive fibers had disappeared, suggesting that most, if not all, NPY-positive fibers in the iris originate in the superior cervical ganglion.

In irides from long-term sympathectomized animals, a high number of TH- and NPY-immunoreactive fibers had reappeared, while such irides were devoid of catecholamine-containing fibers, as evidenced by Falck-Hillarp histochemistry. The appearance of TH- and NPY-positive fibers in sympathetically denervated irides was clearly time dependent. The distribution of fluorescent fibers in irides from intact and sympathectomized animals showed obvious dissimilarities such as a lower fluorescence intensity and fewer varicose fibers in denervated irides. Furthermore, in irides from sympathectomized rats, TH- and NPY-positive fibers were not associated with blood vessels.

Unilateral removal of the parasympathetic ciliary ganglion, which supplies the iris with cholinergic fibers, 3 days prior to sacrifice in animals bilaterally sympathectomized 1 month earlier, led to a drastic reduction in numbers of TH- and NPY-positive iris fibers on the ciliarectomized/sympathectomized side as compared to the sympathectomized-alone side. The present experiments thus suggest that adult cholinergic neurons *in vivo* are capable of expressing adrenergic characteristics under experimental conditions.

Received September 11, 1984;
Accepted October 26, 1984

[1] This work was supported by the Swedish Medical Research Council (Grants 14X-03185, 14P-5867, 12P-7310, 04X-2887, and 13X-1010), Magnus Bergvalls Stiftelse, Knut och Alice Wallenbergs Stiftelse, Karolinska Institutets fonder, and by grants from the National Institute of Neurological and Communicative Disorders and Stroke (Grant 06801) and the National Institute of Mental Health (Grant 02714). We thank Lena Hultgren, Anna Hultgårdh, Barbro Standwerth, and Ingrid Strömberg for technical assistance and Ida Engqvist for secretarial help.

[2] To whom correspondence should be sent, at Department of Histology, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden.

The iris has a rich sympathetic, parasympathetic, and sensory innervation. Sympathetic fibers originate in the superior cervical ganglion and, using Falck-Hillarp histochemistry (Falck, 1962; Malmfors, 1965) or immunohistochemistry with tyrosine hydroxylase (TH) antiserum, a regular, dense, fine-meshed network of fluorescent fibers can be visualized in iris whole mounts. Recently, a population of sympathetic noradrenergic neurons, including cells in the superior cervical ganglion, has been found to contain a substance that reacts with antisera against neuropeptide Y (NPY) (Lundberg et al., 1982b), a peptide recently isolated from porcine brain (Tatemoto, 1982; Tatemoto et al., 1982). NPY-immunoreactive nerve fibers have also been demonstrated in the iris itself with a distribution similar to that of the adrenergic innervation (Terenghi et al., 1982). Surprisingly, Allen et al. (1983) using radioimmunoassay and Terenghi et al. (1983) using immunohistochemistry have reported presence in the iris of NPY, although in reduced amounts, 1 month after sympathectomy. This might suggest that not all NPY-positive iris fibers also contain noradrenaline. An alternative hypothesis is that production of NPY-like material has been induced in nonsympathetic fibers as a response to the sympathectomy. Presence of catecholamine-synthesizing enzymes in cultured cholinergic neurons has been reported (Teitelman, et al., 1984). Similarly, Grzanna and Coyle (1978) have shown that mature cholinergic neurons in the rat submandibulary ganglion contain dopamine-β-hydroxylase without containing noradrenaline. Furthermore, cultured sympathetic neurons from newborn rats can express adrenergic and/or cholinergic characteristics, depending upon the culture medium (for reviews, see Patterson, 1979; Potter et al., 1981; Burton and Bunge, 1981).

The iris is a favorable tissue for studies on interactions between different neuronal populations, their transmitters, and the target tissue itself. For example, sympathectomy has been demonstrated to lead to permanently increased levels of substance P (Kessler et al., 1983a; Allen et al., 1983), a peptide localized to sensory neurons (Cuello et al., 1978; Miller et al., 1981), thus indicating that sympathetic fibers in the iris might normally influence the number and/or state of adult sensory fibers.

In the present study, two problems are addressed. First, we have examined and compared the distribution of NPY- and TH-immunoreactive nerve fibers in normal rat iris, ciliary body, and choroid membrane. The second objective was to investigate whether surgical sympathectomy can lead to induction of adrenergic characteristics in nonsympathetic iris nerve fibers.

Materials and Methods

Approximately 75 adult albino Sprague-Dawley rats (Alab, Stockholm, Sweden) of both sexes were used. Sympathetic denervations were performed bilaterally or unilaterally under deep ether anaesthesia by surgical removal of the superior cervical ganglion (Malmfors, 1965), resulting in a total of 80 denervated eyes available for histochemical analysis. Sympathectomized animals were sacrificed after different postoperative times ranging from 1 day to 4 months. The parasympathetic iris nerves were removed by unilateral extirpation of the ciliary ganglion (Malmfors and Nilsson, 1964) in five animals that had been sympathectomized 1 month earlier. Grafting of irides to the anterior chamber of the eye (Olson and Malmfors, 1970) was performed using four adult rats as recipients and an equal number of animals as donors.

Animals were killed by exsanguination under deep ether anaesthesia. Irides, iris grafts, and ciliary ganglia were fixed in picric acid/formaldehyde (Stefanini et al., 1967; Zamboni and De Martino, 1967) overnight, essentially according to Costa et al. (1980), as described previously (Björklund et al., 1984). Some irides were divided into two or three pieces. The indirect immunofluorescence technique (Coons, 1958) was applied to floating specimens overnight at 4°C. Antisera to TH (Markey et al., 1980) and NPY (Lundberg et al., 1984) were raised in rabbits and used diluted 1:200 (TH and NPY) or 1:400 (NPY) in phosphate-buffered saline. After rinsing in the buffer three times for 10 min, specimens were incubated in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin antibodies (Dakopatts Denmark) diluted 1:50 or 1:100 for 60 min at room temperature in darkness. After a second rinse, the specimens were mounted on gelatin-coated glass slides in a mixture of glycerol and phosphate buffer (9:1).

Due to their small size, ciliary ganglia are difficult to section. Therefore, after fixation and incubation in primary and secondary antisera, such ganglia from intact rats and rats sympathectomized 2 days and 3 months earlier were dried to slides in the same way as irides.

Specimens were examined in a darkfield fluorescence microscope. In several experiments, the amount of fluorescent fibers was semiquantitatively estimated using coded slides. The scale used ranged from 0 to 5 with ½ between steps, giving a total of 11 steps.

Although the antisera used have been characterized, the technique does not allow absolute identification of TH and NPY, respectively. Therefore, positive findings should be interpreted as "TH- and NPY-like" immunoreactivity.

Results

Intact irides. Using TH-antiserum, a high number of varicose, strongly fluorescent fibers were seen forming a regular network in the iris dilator area and in the sphincter region (Fig. 1*a*). Few smooth positive fibers could be seen running together with otherwise negative fibers in major bundles. The distribution and amount of fluorescent fibers were identical to that seen with Falck-Hillarp histochemistry. Thus, a prominent blood vessel innervation was readily observed (Fig. 1b). The ciliary body was richly innervated by TH-positive fibers. Such fibers were also found in the choroid membrane.

A system of varicose and smooth fibers similar to the TH-positive system was also visualized uing NPY-antiserum (Fig. 2*a*), including, again, a prominent blood vessel innervation (Fig. 2*b*) and fibers in the sphincter. The total amount of NPY-positive fibers seemed to be slightly lower than that observed using TH-antiserum. A more prominent difference was, however, the clearly weaker fluorescence intensity obtained with NPY-antiserum as compared to TH-antiserum. A high density of varicose NPY-immunoreactive nerve fibers was also present in the choroid membrane where they could frequently be observed along blood vessels (Fig. 2*c*).

Intraocular iris grafts. In iris grafts examined 2 days after transplantation, no NPY-positive fibers could be detected, and only some few smooth TH-positive fibers could be detected. These latter fibers were axons probably still not phagocytozed. In contrast, in 2-month-old iris grafts, a regular network of relatively strongly fluorescent varicose fibers could be visualized using NPY-antiserum (Fig. 2*f*). These fibers had a relatively organotypic distribution.

Sympathetically denervated irides. Irides examined 1 or 2 days after surgical removal of the superior cervical ganglion were almost totally devoid of the normally occurring TH- and NPY-positive networks (Figs. 2*c* and 3*d*). However, they usually contained a couple of individual fluorescent, varicose fibers seen using either TH- or NPY-antisera. A dense network of very weakly fluorescent, thus barely detectable, fibers was also observed with both antisera, although less clearly with the NPY-antiserum. Six days after denervation, an increased number of still relatively weakly fluorescent fibers was observed. They were more prominent with TH- than with NPY-antiserum.

The amount of visible TH-fibers and their fluorescence intensity continued to gradually increase as a function of time after sympathectomy (Figs. 1 and 3). Thus, at 1 month, and even more pronounced at 4 months postoperatively, a dense regular plexus of strongly fluorescent TH-immunoreactive fibers was observed (Fig. 1, *e* and *f*). The total amount of fluorescent fibers seemed to be comparable to that seen in normal irides. However, several characteristics distinguished the TH-positive iris fiber plexus in chronically sympathectomized animals from that in normal rats. Thus, although the fluorescence intensity was relatively high in the chronically denervated irides, it never reached the intensity seen in normal ones. Similarly, although many varicose fibers could be observed, the ratio of smooth/varicose fibers was higher in the chronically denervated irides. In addition, an increased number of smooth fluorescent fibers were observed in the fiber bundles of denervated specimens. Normally, only very few positive fibers were present in these bundles. Finally, in striking contrast to normal irides, no blood vessel innervation could be detected following sympathectomy.

Although a gradual increase in amount of fluorescent fibers could be observed also using NPY-antiserum (Fig. 4), the increase was less pronounced than with TH-antiserum. Thus, when sympathectomized irides from different time points were compared, there were always both fewer and less strongly fluorescent NPY-positive fibers as compared to TH-positive ones. However, the differences in distribution between irides from normal and long-term sympathectomized animals were similar with both antisera. To ensure that the presence of TH- and NPY-immunoreactive iris fibers in chronically denervated rats was not due to incomplete sympathectomies, five such irides from animals operated 2 months earlier were divided into three pieces, one reacted for Falck-Hillarp histochemistry and the other two for TH- and NPY-immunohistochemistry, respectively. As expected, all irides contained TH- and NPY-positive fibers, while no fluorescent fibers could be seen in the pieces reacted for Falck-Hillarp histochemistry (Fig. 5).

Effect of ciliarectomy on TH- and NPY-positive fibers in long-term sympathetically denervated irides. Unilateral surgical removal of the parasympathetic ciliary ganglion 3 days prior to sacrifice in five animals bilaterally sympathectomized 2 months earlier drastically reduced the number of both TH- and NPY-positive fibers on the ciliarectomized side as compared to the only sympathectomized control irides (Figs. 6 and 7). Some of the ciliarectomized irides were more or less completely devoid of TH-positive fibers, whereas others contained variable fiber numbers, although always substantially fewer than the controls. A low number of NPY-positive fibers could be detected in all ciliarectomized irides. Semiquantitative estimations showed the differences in numbers of TH- and NPY-positive fibers to be highly significant between experiment and control irides (Fig. 7).

All ciliary ganglia had a relatively high background fluorescence. Fluorescent fibers, as well as cell bodies, were observed in all specimens. When fluorescence intensity of the perikarya was estimated on a blind basis, there was a significant difference between ganglia from short-term sympathectomized rats on the one hand and ganglia from control and long-term sympathectomized rats on the other. Thus, the ciliary neurons in short-term sympathectomized rats seemed to have an enhanced TH-immunoreactivity.

Discussion

Coexistence of a classical neurotransmitter with one or more neuropeptides is by now a well-established phenomenon (Hökfelt et

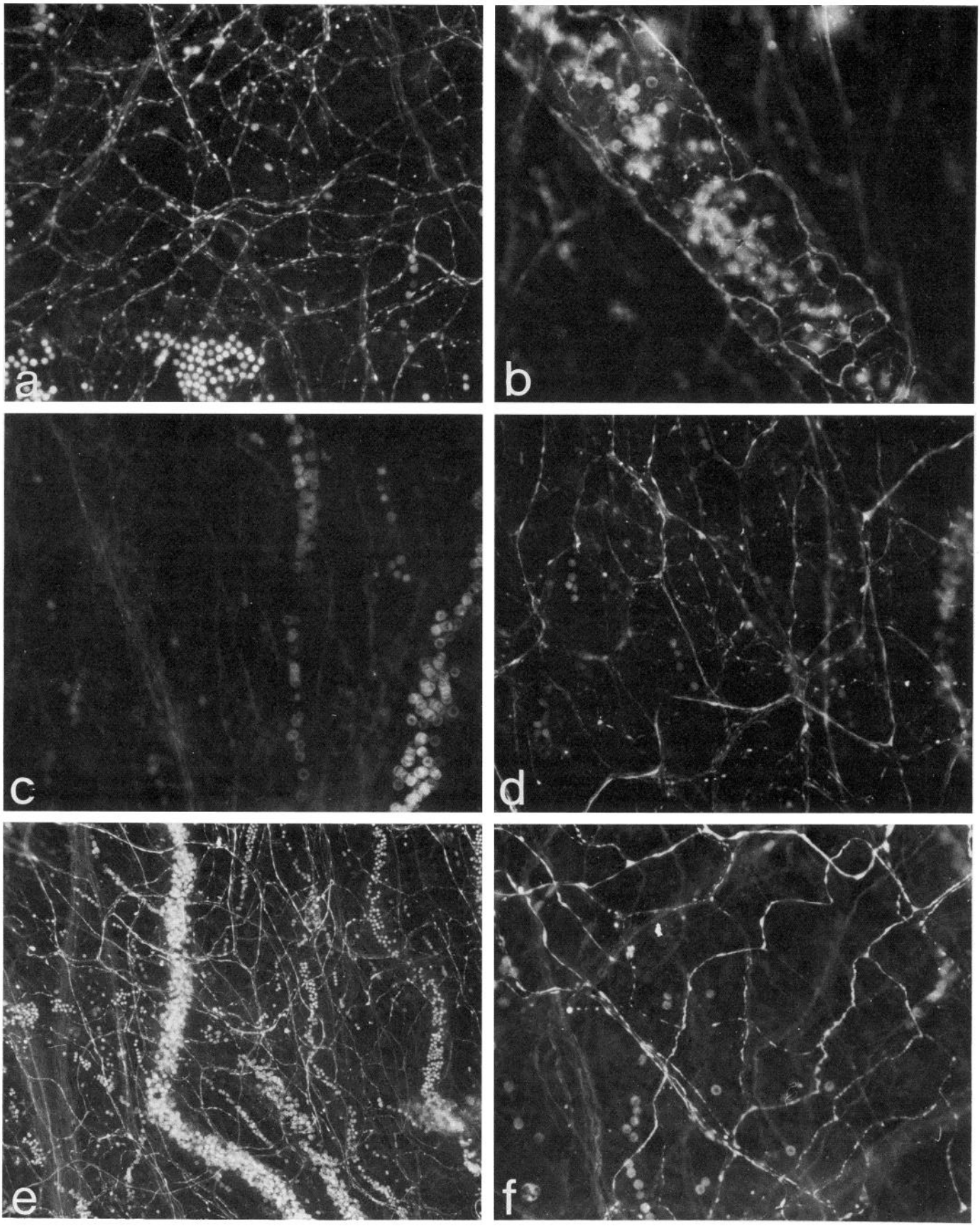

Figure 1. TH-like immunoreactivity. *a*, Regular plexus of TH-positive fibers in a normal rat iris. *b*, Prominent blood vessel innervation seen in such irides. *c*, Almost total lack of fluorescence seen in irides from animals sympathectomized 1 day earlier. *d*, Ten days after a sympathectomy, a relatively high number of brightly fluorescent fibers can be observed. *e* and *f*, Four months after sympathectomy, the amount and fluorescence intensity of TH-positive fibers is even more prominent. Magnification: *a* to *d*; and *f*, × 330; *e*, × 135.

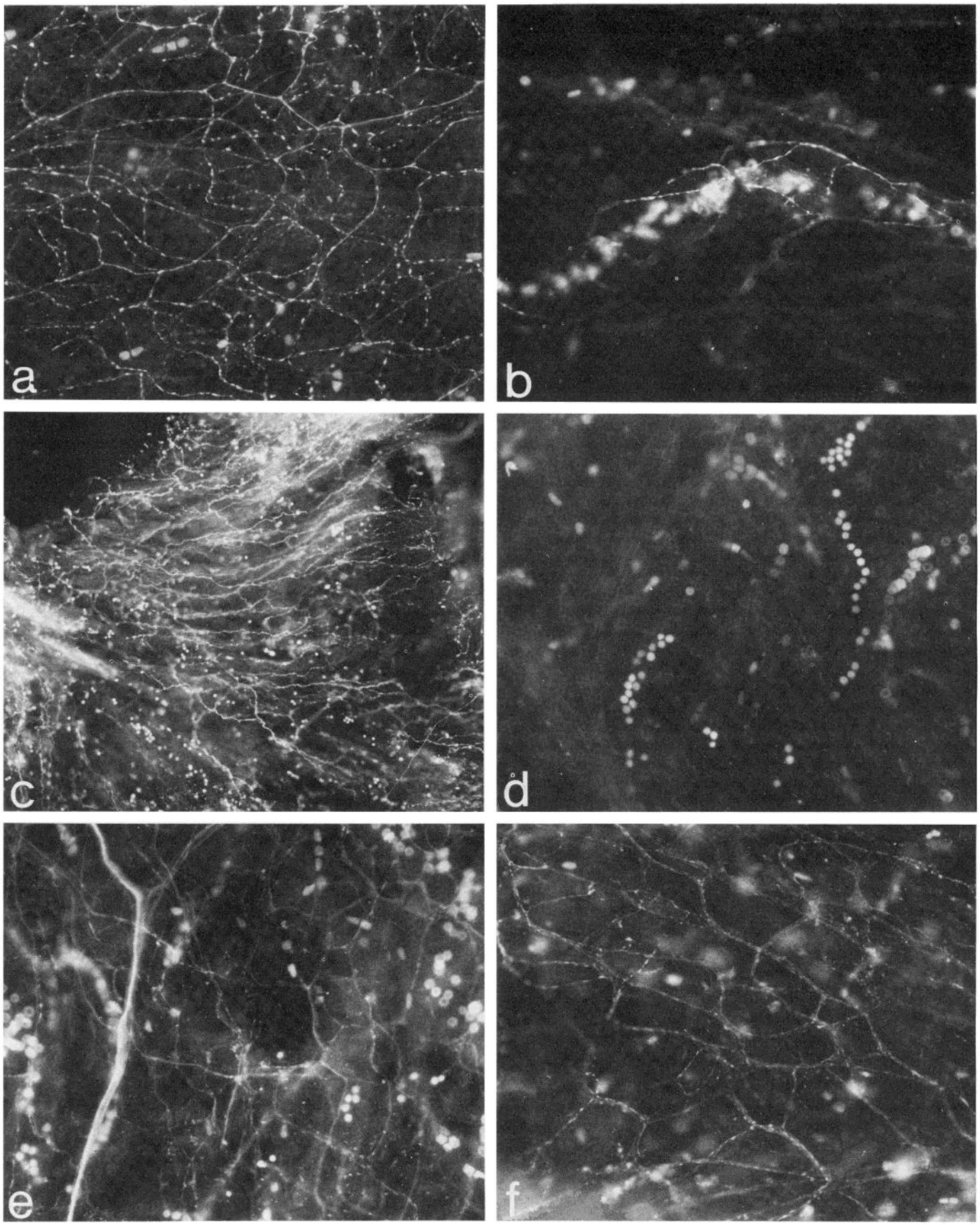

Figure 2. NPY-like immunoreactivity. *a* and *b*, A fiber system similar to that seen with TH-antiserum is observed in normal irides (*a*) including blood vessel innervation (*b*). *c*, A high number of fluorescent fibers is present in the choroid membrane of normal rats. *d*, Almost total lack of NPY-positive iris fibers 2 days after a sympathectomy, whereas in *e*, a high number of brightly fluorescent fibers is present 1 month postoperatively. *f*, NPY-positive fibers in a whole mounted intraocular iris graft 2 months after transplantation. Magnification × 330.

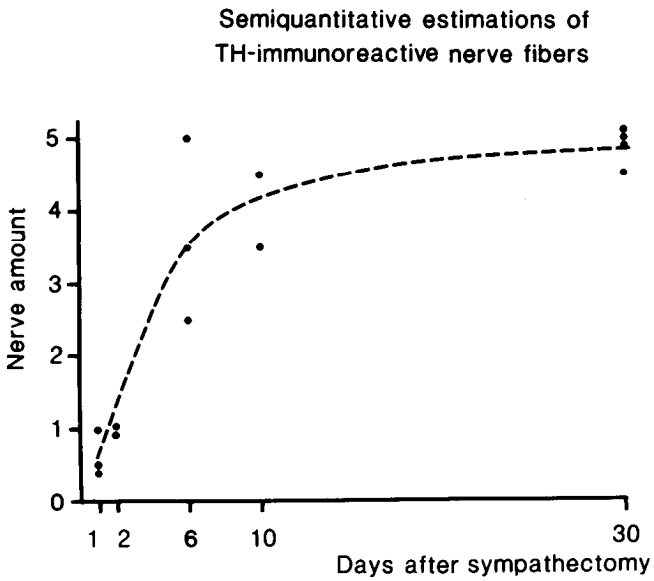

Figure 3. Amount of TH-positive nerve fibers in whole-mounted irides from sympathectomized animals after various postoperative times. A clearly time-dependent appearance of TH-immunoreactive fibers is seen. Each *dot* represents one iris. Semiquantitative estimations performed on coded slides.

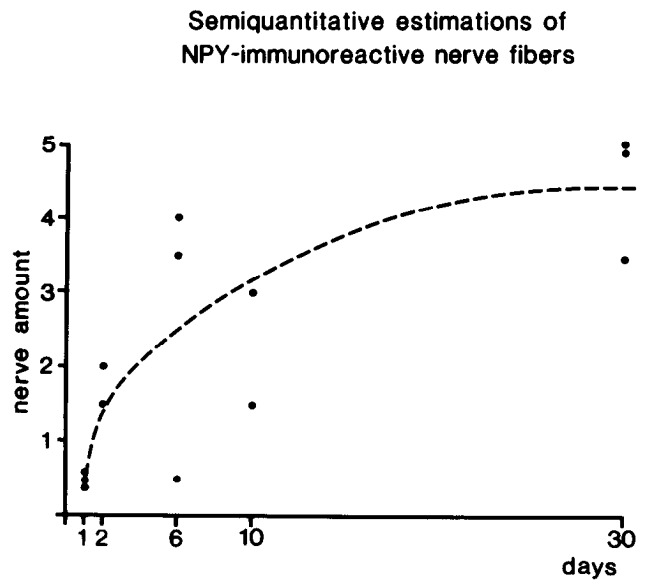

Figure 4. Amount of NPY-positive nerve fibers in whole mounted irides from sympathectomized animals after various postoperative times. As with TH antiserum, the amount of fluorescent fibers shows a gradual increase with time after sympathectomy. Each *dot* represents one iris. Semiquantitative estimations performed on coded slides.

al., 1980, 1982; Cuello, 1982; Lundberg et al., 1982a; Chan-Palay and Palay, 1983). NPY, a 36-amino acid peptide and a member of the pancreatic polypeptide family (Tatemoto et al., 1982), is present in widely distributed systems in both the central and peripheral nervous system (Everitt et al., 1984; Lundberg et al., 1982b; Hökfelt et al., 1984; Terenghi et al, 1983; Gu et al., 1983; Furness et al., 1983; Edvinsson et al, 1983; Stjernquist et al., 1983; see also Emson and DeQuidt, 1984) and is, in several places, colocalized with catecholamines (Everitt et al., 1984, Lundberg et al., 1982a, Hökfelt et al., 1984).

Using immunohistochemistry on iris whole mounts, we now report that NPY is present in a dense regular fiber network in the rat iris and choroid membrane. With antiserum against TH, a very similar fiber system, including fibers surrounding blood vessels, was visualized. Our results agree with those of Terenghi et al. (1983), suggesting that NPY is at least partly present in adrenergic iris nerve fibers originating in the superior cervical ganglion. The density of positive fibers seemed to be lower using NPY-antiserum as compared to TH-antiserum. Since not all catecholamine cell bodies in the superior cervical ganglion contain NPY (Lundberg et al., 1982b), the same may hold true for terminals in the iris. However, the difference in density between NPY- and TH-immunoreactive fibers must be interpreted with caution. Levels of NPY might be variable and in some fibers, and indeed cell bodies, too low to allow immunohistochemical detection. The fluorescence intensity was also clearly weaker using NPY-antiserum as compared to TH-antiserum. The possibility that a few NPY-containing fibers in the normal rat iris originate from other cell bodies than those in the superior cervical ganglion cannot be fully excluded.

One and 2 days after sympathectomy, a couple of relatively strongly fluorescent NPY-positive fibers can often be observed. Whether they represent not-yet-phagocytozed degenerating sympathetic fibers or healthy nonsympathetic fibers cannot be determined. Similarly, a few strongly fluorescent TH-positive fibers were also detected. It should be noted that such irides are completely devoid of noradrenergic fibers as determined by Falck-Hillarp histochemistry. It is, however, safe to conclude that the large majority (approximately 99%) of NPY-iris fibers originate in the superior cervical ganglion.

The gradual appearance with time of fibers expressing TH- and NPY-like immunoreactivity in sympathectomized irides, in which, at the same time, there is no reappearance of catecholamine-containing fibers as judged from parallel anaylsis using formaline-induced fluorescence, is interesting. We have not observed signs of active fiber growth, such as growth cones, in any of the sympathectomized irides studied with NPY- and TH-antisera. Regrowth of sympathetic catecholamine-containing fibers does not occur (see Olson and Malmfors, 1970). Instead, it is our impression that other normally present fibers gradually increase their content of TH- and NPY-like substances and thus become immunohistochemically detectable. The fact that a significant reduction in amount of TH- and NPY-positive fibers was observed in chronically sympathectomized irides after short-term ciliarectomy of course strongly suggests that at least a large part of these fibers are cholinergic. Whether other types of nerve fibers reaching the iris e.g. via the ciliary ganglion also contribute to the population of TH- and NPY-positive fibers in chronically sympathectomized rats or whether the persistence of some fibers was due to incomplete ciliarectomies is difficult to determine.

There are several examples of cholinergic neurons expressing adrenergic characteristics. Using chick embryos, Teitelman et al. (1984) have reported that most ciliary neurons *in vitro* expresses TH- and also L-aromatic-acid-decarboxylase and phenylethanolamine-*N*-methyl-transferase, while only a few cells *in vivo* contain TH. Furthermore, the presence of dopamine-β-hydroxylase in parasympathetic submandibular ganglion cells has been observed in the adult rat *in vivo* (Grzanna and Coyle, 1978). The most thoroughly characterized example of adrenergic-cholinergic plasticity is, however, that which occurs *in vitro* during development of superior cervical ganglion neurons. Immediately after plating, such cells express only adrenergic characteristics such as small granular vesicles indicative of stored noradrenaline (Johnson et al., 1976, 1980; Landis et al., 1980) and catecholamine synthesis (Patterson and Chun, 1977b). However, when cultured in the presence of certain non-neuronal cells (Patterson and Chun 1974; Patterson et al., 1975) or in conditioned medium from such cells (Patterson and Chun, 1977a), the cells acquire different cholinergic properties such as choline acetyltransferase activity (Patterson and Chun, 1977a; Iacovitti et al., 1981), synthesis and storage of acetylcholine (Patterson and Chun, 1974, 1977a), and formation of cholinergic synapses (O'Lague et al., 1978; Landis, 1980). At the same time, adrenergic properties are gradually lost. In between, the neurons, for a while, are dual in function (Furshpan et al., 1976), expressing both adre-

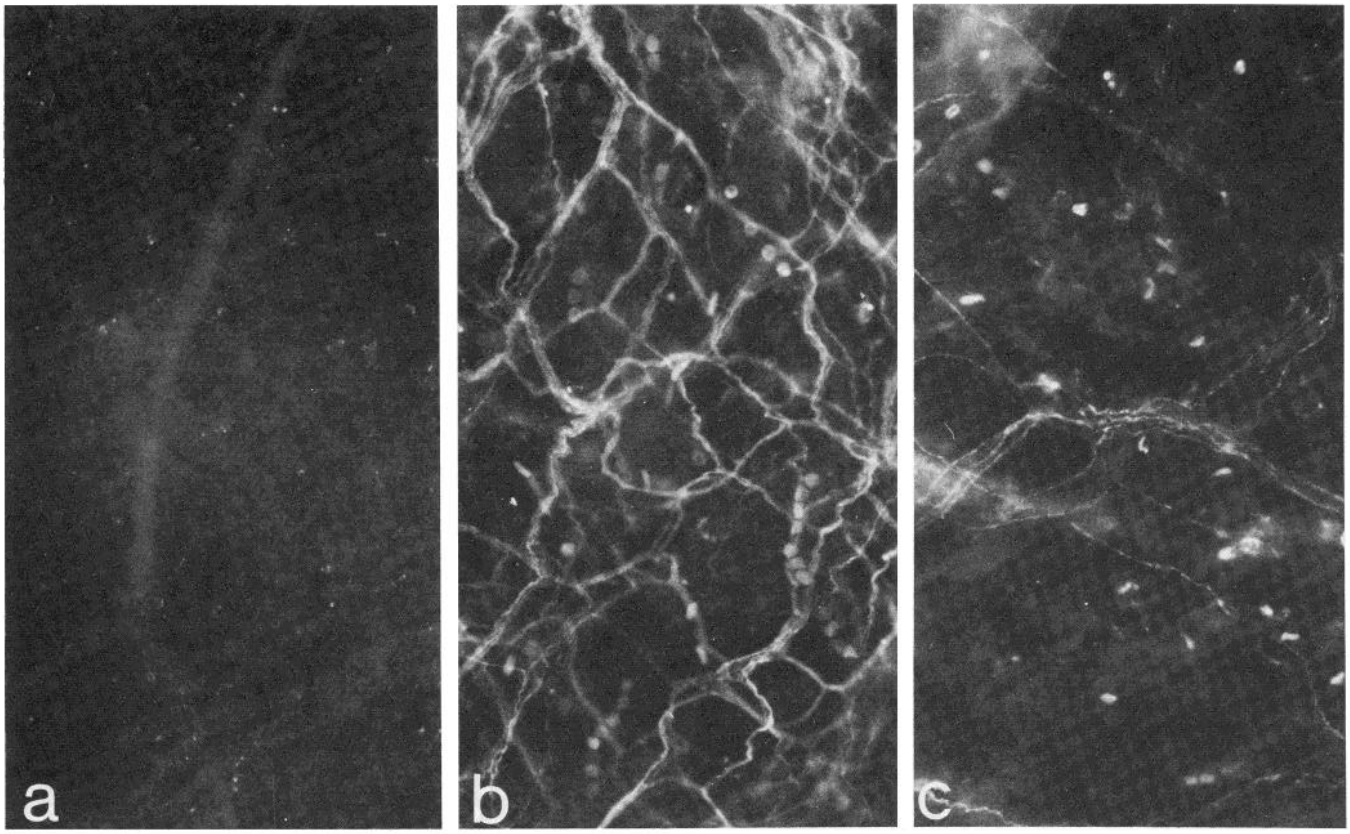

Figure 5. *a* to *c* are three parts of one iris from an animal sympathectomized 2 months earlier. *a*, Falck-Hillarp histochemistry; *b*, TH-immunohistochemistry; and *c*, NPY-immunohistochemistry. No fibers can be seen in *a*, whereas both *b* and *c* contain a large amount of brightly fluorescent fibers. Magnification × 330.

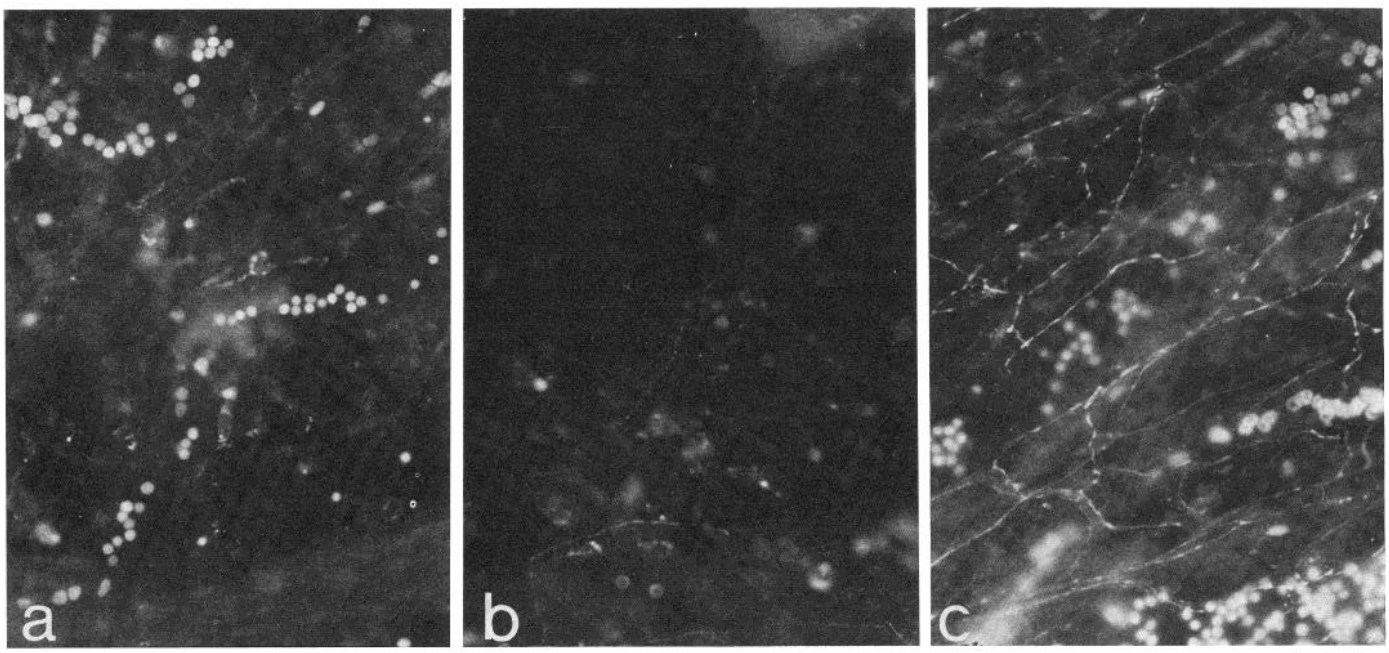

Figure 6. Effect of ciliarectomy on TH- and NPY-positive fibers in long-term sympathetically denervated irides. *a* and *b* show the almost complete lack of TH- (*a*) and NPY (*b*) -positive fibers in irides ciliarectomized 3 days and sympathectomized 1 month before sacrifice. *c* illustrates the abundant number of NPY-positive fibers in the iris from the contralateral, only sympathectomized side. Magnification × 330.

nergic and cholinergic properties (Potter et al., 1981; Higgins et al., 1981; Iacovitti et al., 1981; Furshpan et al., 1982). A similar phenomenon has recently been described *in vivo*, where sympathetic fibers innervating sweat glands of the rat footpad lose their endogenous catecholamine content as they mature into the cholinergic phenotype (Landis, 1980; Landis and Keefe, 1980, 1983; Siegel et al., 1982), while the capacity to take up catecholamines is partly retained. We have now shown that also *mature cholinergic* fibers *in vivo* can

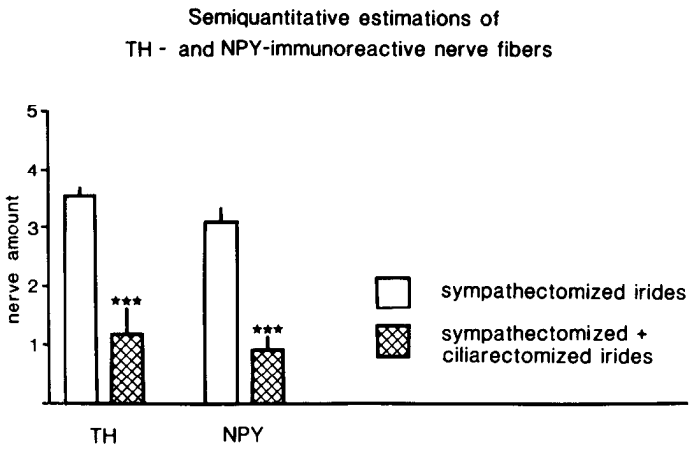

Figure 7. Comparison of amount of TH- and NPY-immunoreactive nerve fibers in irides from animals bilaterally sympathectomized 1 month and unilaterally ciliarectomized 3 days before sacrifice. *Each bar* represents the mean of four to five observations performed on coded slides. ***, $p \geq 0.001$.

express TH- and NPY-like immunoreactivity. Thus, it seems as if both mature and immature sympathetic and parasympathetic neurons are able to express both cholinergic and adrenergic characteristics.

The mechanism regulating increases in TH- and NPY-like substances in nonadrenergic iris nerves following sympathectomy is intriguing. Sympathetic denervation has been demonstrated to lead to increased levels of substance P in the rat iris (Kessler et al., 1983a, b; Allen et al., 1983). Interestingly, this effect seems to be mediated via nerve growth factor (NGF), since exogenously applied NGF mimics the effect of sympathectomy, whereas anti-NGF prevents it (Kessler et al., 1983b). Although survival and growth of parasympathetic neurons is not NGF-dependent, it is possible that NGF might influence other cell functions such as expression of transmitter characteristics. It is also noteworthy that iris substance-P levels increase slowly after sympathectomy (Cole et al., 1983), thereby parallelling the gradual appearance of TH- and NPY-positive fibers reported in this study. It is, of course, possible that sympathectomy leads to increased availability in the iris of other as-yet-unknown substances which stimulate expression of TH- and NPY-like immunoreactivity in nonsympathetic iris nerves. The fact that very weak fluorescent fibers can be seen with TH- and NPY-antisera already one day after sympathectomy could indicate that TH- and NPY-like substances may normally be produced in low, immunohistochemically nondetectable levels in nonsympathetic nerves and that the adrenergic innervation normally prevents full expression of such properties.

Whether the increases of TH- and NPY-like substances in nonsympathetic fibers following sympathectomy is of any functional importance for the animal is unknown. Apparently, catecholamines do not seem to be stored in these fibers. Landis and Keefe (1983) have also failed to detect catecholamine fluorescence after exogeneous administration of α-methylnoradrenaline in irides from rats sympathetically denervated 4 days earlier. If, however, NPY is actually released, one might speculate that this, perhaps together with circulating catecholamines, might exert local effects in the iris musculature.

In conclusion, we report that normal rat iris, ciliary body, and choroid membrane contain a high number of NPY-positive nerves with a distribution similar to the adrenergic innervation. Furthermore, in surgically sympathectomized rats, TH- and NPY-immunoreactivity gradually appears in nonsympathetic iris fibers. A large part of these fibers seem to be originating in the ciliary ganglion. Since this ganglion provides the iris with cholinergic fibers, we conclude that adult cholinergic neurons *in vivo* can be induced to express adrenergic characteristics.

References

Allen, J. M., G. P. McGregor, T. E. Adrian, S. R. Bloom, S. Q. Zhang, K. W. Ennis, and W. G. Unger (1983) Reduction of neuropeptide Y (NPY) in the rabbit iris-ciliary body after chronic sympathectomy. Exp. Eye Res. *37:* 213–215.

Björklund, H., B. Hoffer, L. Olson, M. Palmer, and Å. Seiger (1984) Enkephalin immunoreactivity in iris nerves: Distribution in normal and grafted irides, persistence and enhanced fluorescence after denervations. Histochemistry *80:* 1–7.

Burton, H., and R. P. Bunge (1981) The expression of cholinergic and adrenergic properties by autonomic neurons in tissue culture. In *Excitable Cells in Tissue Culture*, P. G. Nelson and M. Lieberman, eds., pp. 1–36, Plenum Press, New York.

Chan-Palay, V., and S. L. Palay (1983) *Coexistence of Neuroactive Substances in Neurons*, Wiley Interscience, New York.

Cole, D. F., S. R. Bloom, G. Burnstock, J. M. Butler, G. P. McGregor, M. J. Saffrey, W. G. Unger, and S. Q. Zhang (1983) Increase in SP-like immunoreactivity in nerve fibers of rabbit iris and ciliary body one to four months following sympathetic denervation. Exp. Eye Res. *37:* 191–197.

Coons, A. H. (1958) Fluorescent antibody methods. In *General Cytochemical Methods*, J. F. Danielli, ed., pp. 399–422, Academic Press, Inc., New York.

Costa M., R. Buffa, J. B. Furness, and E. Solcia (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. Histochemistry *65:* 157–165.

Cuello, A. C. (1982) *Co-Transmission*, MacMillan, Basingstoke.

Cuello, A. C., M. del Fiacco, and G. Paxinos (1978) The central and peripheral ends of the substance P-containing sensory neurons in the rat trigeminal system. Brain Res. *152:* 499–509.

Edvinsson, L., P. Emson, J. McCulloch, K. Tatemoto, and R. Uddman (1983) Neuropeptide Y: Cerebrovascular innervation and vasomotor effects in the cat. Neurosci. Lett. *43:* 79–84.

Emson, P. C. and M. E. DeQuidt (1984) NPY—A new member of the pancreatic polypeptide family. Trends Neurosci. *7:* 31–35.

Everitt, B. J., T. Hökfelt, L. Terenius, K. Tatemoto, V. Mutt, and M. Goldstein (1984) Differential coexistence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience *11:* 443–462.

Falck, B. (1962) Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta Physiol. Scand. *56* (*Suppl.* 197): 1–25.

Furness, J. B., M. Costa, P. C. Emson, R. Håkanson, E. Moghimzadeh, F. Sundler, I. L. Taylor, and R. E. Chance (1983) Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. Cell Tissue Res. *234:*; 71–92.

Furshpan, E. J., P. R. MacLeish, P. H. O'Lague, and D. D. Potter (1976) Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures. Evidence for cholinergic, adrenergic, and dual function neurons. Proc. Natl. Acad. Sci. U. S. A. *73:* 4225–4229.

Furshpan, E. J., D. D. Potter, and S. C. Landis (1982) On the transmitter repertoire of sympathetic neurons in culture. Harvey Lect. *76:* 149–191.

Grzanna, R., and J. T. Coyle (1978) Dopamine-β-hydroxylase in rat submandibular ganglion cells which lack norepinephrine. Brain Res. *151:* 206–214.

Gu, J., J. M. Polak, T. E. Adrian, J. M. Allen, K. Tatemoto, and S. R. Bloom (1983) Neuropeptide tyrosine (NPY)—a major cardiac neuropeptide. Lancet *1:* 1008–1010.

Higgins, D., L. Iacovitti, T. H. Joh, and H. Burton (1981) The immunocytochemical localization of tyrosine hydroxylase within rat sympathetic neurons that release acetylcholine in culture. J. Neurosci. *1:* 126–131.

Hökfelt, T., O. Johansson, Å. Ljungdahl, J. M. Lundberg, and M. Schultzberg (1980) Peptidergic neurons. Nature *284:* 515–521.

Hökfelt, T., J. M. Lundberg, L. Skirboll, O. Johansson, M. Schultzberg, and S. R. Vincent (1982) Coexistence of classical transmitters and peptides in neurons. In *Co-transmission*, A. C. Cuello, ed., pp. 77–126, MacMillan, London.

Hökfelt, T., O. Johansson, and M. Goldstein (1984) Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. In *Handbook of Chemical Neuroanatomy*, Vol. 2, Classical Transmitters in the CNS, Part I, A. Björklund and T. Hökfelt, eds., pp 157–276, Elsevier-North Holland Publishing Co., Amsterdam.

Iacovitti, L., T. H. Joh, D. H. Park, and R. P. Bunge (1981) Dual expression of neurotransmitter synthesis in cultured neurons. J. Neurosci. *1:* 685–690.

Johnson, M., D. Ross, M. Meyers, R. Rees, R. Bunge, E. Wakshull, and H. Burton (1976). Synaptic vesicle cytochemistry changes when cultured sympathetic neurons develop cholinergic interactions. Nature *262:* 308–310.

Johnson, M., C. D. Ross, M. Meyers, E. L. Spitznagel, and R. P. Bunge (1980) Morphological and biochemical studies on the development of cholinergic properties in cultured sympathetic neurons I. Correlative changes in choline acetyltransferase and synaptic vesicle cytochemistry. J. Cell Biol. *84:* 680–691.

Kessler, J. A., W. O. Bell, and I. B. Black (1983a) Substance P levels differ in sympathetic target organ terminals and ganglion perikarya. Brain Res. *258:* 114–116.

Kessler, J. A., W. O. Bell, and I. B. Black (1983b) Interactions between the sympathetic and sensory innervation of the iris. J. Neurosci. *3:* 1301–1307.

Landis, S. C. (1980) Developmental changes in the neurotransmitter properties of dissociated sympathetic neurons: A cytochemical study of the effects of medium. Dev. Biol. *77:* 348–361.

Landis, S. C. (1983) Development of cholinergic sympathetic neurons: Evidence for transmitter plasticity *in vivo*. Fed. Proc. *42:* 1633–1638.

Landis, S. C., and D. Keefe (1980) Development of cholinergic sympathetic innervation of eccrine sweat glands in rat foot pad. Society for Neuroscience Annual Meeting Conference Report (Brain Information Service, Los Angeles) *10:* Abstract No. 131.20.

Landis, S. C., and D. Keefe (1983) Evidence for neurotransmitter plasticity *in vivo*: Developmental changes in properties of cholinergic sympathetic neurons. Dev. Biol. *98:* 349–372.

Lundberg, J. M., B. Hedlund, A. Änggård, J. Fahrenkrug, T. Hökfelt, K. Tatemoto, and T. Bartfai (1982a) Co-storage of peptides and classical transmitters in neurons. In *Systemic Role of Regulatory Peptides*, S. R. Bloom, J. M. Polak, and E. Lindenlaub, eds., pp. 93–119, Schottauer, Stuttgart.

Lundberg, J. M., L. Terenius, T. Hökfelt, C. R. Martling, K. Tatemoto, V. Mutt, J. Polak, S. Bloom, and M. Goldstein (1982b) Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. Acta Physiol. Scand. *116:* 477–480.

Lundberg, J. M., L. Terenius, T. Hökfelt, and K. Tatemoto (1984) Comparative immunohistochemical and biochemical analysis of polypeptide-like peptides with special reference to presence of neuropeptide Y in central and peripheral neurons. J. Neurosci. *4:* 2376–2386.

Malmfors, T. (1965) Studies on adrenergic nerves. The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels. Acta Physiol. Scand. (Suppl.) *248:* 1–93.

Malmfors, T., and O. Nilsson (1964) Parasympathetic postganglionic denervation of the iris and the parotid gland in the rat. Acta Morphol. Neerl.-Scand. *6:* 81–85.

Markey, K. A., S. Kondo, I. Shenkman, and M. Goldstein (1980) Purification and characterization of tyrosine hydroxylase from a clonal phaeochromocytoma cell line. Mol. Pharmacol. *17:* 79–85.

Miller, A., M. Costa, J. B. Furness, and I. W. Chubb (1981) Substance P-immunoreactive sensory nerves supply the rat iris and cornea. Neurosci. Lett. *23:* 243–249.

Olson, L., and T. Malmfors (1970) Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and [^{3}H]noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. Acta Physiol. Scand. (Suppl.) *348:* 1–112.

O'Lague, P. H., D. D. Potter, and E. J. Furshpan (1978) Studies on rat sympathetic neurons developing in cell culture. III. Cholinergic transmission. Dev. Biol. *67:* 424–443.

Patterson, P. H. (1979) Environmental determination of neurotransmitter functions in developing sympathetic neurons. Prog. Brain Res. *51:* 75–83.

Patterson, P. H., and L. L. Y. Chun (1974) The influence of non-neuronal cells on catecholamine and acetylcholine synthesis and accumulation in cultures of dissociated sympathetic neurons. Proc. Natl. Acad. Sci. U. S. A. *71:* 3607–3610.

Patterson, P. H., and L. L. Y. Chun (1977a) The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. Dev. Biol. *56:;* 263–280.

Patterson, P. H., and L. L. Y. Chun (1977b) The induction of acetylcholine synthesis in primary cultures of dissociated sympathetic neurons II. Developmental aspects. Dev. Biol. *60:* 473–481.

Patterson, P. H., L. F. Reichardt, and L. L. Y. Chun (1975) Biochemical studies on the development of primary sympathetic neurons in cell cultures. Cold Spring Harbor Symp. Quant. Biol. *40:* 389–398.

Potter, D. D., S. C. Landis, and E. J. Furshpan (1980) Adrenergic-cholinergic dual function in cultured sympathetic neurons of the rat. Ciba Found. Symp. *83:* 123–138.

Siegel, R., M. Schwab, and S. C. Landis (1982) Development changes in the neurotransmitter properties of cholinergic sympathetic neurons *in vivo*. Soc. Neurosci. Abstr. *8:* 7.

Stefanini, M., C. DeMartino, and C. Zamboni (1967) Fixation of ejaculated spermatozoa for electron microscopy. Nature *216:;* 173–174.

Stjernquist, M., P. Emson, Ch. Owman, N. -O. Sjöberg, F. Sundler, and K. Tatemoto (1983) Neuropeptide Y in the female reproductive tract of the rat. Distribution of nerve fibers and motor effects. Neurosci. Lett. *39:* 279–284.

Tatemoto, K. (1982) Neuropeptide Y: The complete amino acid sequence of the brain peptide. Proc. Natl. Acad. Sci. U. S. A. *79:* 5485–5489.

Tatemoto, K., M. Carlquist, and V. Mutt (1982) Neuropeptide Y—a novel brain peptide with structural similarities for peptide YY and pancreatic polypeptide. Nature *296:* 659–660.

Teitelman, G., L. Iacovitti, T. H. Joh, and D. J. Reis (1984) Plasticity of catecholamine enzyme expression in development. In *Abstracts of the VIIth International Congress of Histochemistry and Cytochemistry, Helsinki, Finland*, p. 430.

Terenghi G., J. M. Polak, L. Probert, G. P. McGregor, G. L. Fein, M. A. Blank, J. M. Butler, W. G. Unger, S. -Q. Zhang, D. F. Cole, and S. R. Bloom (1982) Mapping, quantitative distribution and origin of substance P- and VIP-containing nerves in the uvea of guinea pig eye. Histochemistry *75:* 399–417.

Zamboni, L., and C. DeMartino (1967) Buffered picric acid formaldehyde: A new rapid fixative for electron microscopy. J. Cell Biol. *148A:* 35.