A number of peptides have in recent years attracted attention as putative neurotransmitters or neuromodulators. Many of them were isolated originally from the pancreas or the gastrointestinal tract and were shown to occur also in the brain. Several of them turned out to be alpha-amidated at the C-terminus, and this prompted Tatemoto and Mutt 1 to search for additional such peptides in extracts from pig gut and brain. A peptide with 36 amino acid residues, named neuropeptide Y (NPY), was found to be present in significant amounts in the brain of several animal species. 23 It also was found in many peripheral tissues where it had distinct, biologic effects. 4-8 NPY shows considerable structural resemblance with pancreatic polypeptide and peptide YY, and it was proposed that these peptides form a special family of regulatory peptides. 2 The amino acid sequences of these peptides have been published.9

Neuropeptide Y (NPY) is a recently discovered, amidated 36 amino acid residue neuropeptide present in many but not all sympathetic noradrenergic neurons. In the guinea-pig eye, NPY immunoreactive fibers were found to have the same distribution as noradrenergic fibers except that there were fewer at the iris dilator, in the cornea, and in the chamber angle. In the anterior uvea, the NPY immunoreactive fibers disappeared after excision of the homolateral superior cervical sympathetic ganglion, whereas in the choroid, many NPY immunoreactive fibers remained, indicating that they originate elsewhere. NPY immunoreactivity thus is not found in all sympathetic adrenergic neurons nor is it found only in such nerve fibers. In the retina, NPY immunoreactive fibers formed a single layer of processes in sublamina 1 of the inner plexiform layer. NPY immunoreactive cell bodies were found in the innermost cell row of the inner nuclear layer. The immunoreactivity was concentrated to the hillock region of these cells. Invest Ophthalmol Vis Sci 25:1113-1123, 1984

NPY has been shown to occur in certain sympathetic, noradrenergic neurons.6,8 The internal eye muscles are classical organs for studies on the autonomic nervous system, but the distribution of NPY in them is unknown. We therefore have examined the distribution and origin of nerve fibers with NPY immunoreactivity in the uvea of the guinea-pig eye. Further, the retina is an extensively analyzed and easily accessible piece of CNS tissue that contains several other neuropeptides,10,11 and we therefore have included it in the study.

Materials and Methods

The eyes of 10 adult (300-400 g) outbred guinea-pigs of both sexes were used. Most animals were pigmented, but a few albinotic ones also were examined because the dark melanophores in the pigmented eyes could be suspected to disguise some nerve fibers. However, no differences were discernible between pigmented and albinotic eyes.

Sympathetic denervation was achieved in three animals by excision of the cervical sympathetic ganglion chain on one side, all the way up to the base of the skull. The other side served as control.

The animals were killed by an overdose of diethyl ether, and the eyes were dissected out rapidly, bisected and fixed for 24 hr at +4°C in 4% formaldehyde buffered to pH 7.2 in a 0.1 M phosphate buffer. The
tissue pieces were washed at +4°C for 2 days in several changes of the phosphate buffer containing 30% sucrose. Fifteen micrometer sections were obtained in a cryostat, melted on the microscope slides, and air-dried.

The NPY immunoreactivity was demonstrated with the indirect immunofluorescence method of Coons et al. The NPY antiserum (code no. NPYY/2, used in dilution 1:400) was a kind gift from Dr. Piers Emson, MRC Neuropharmacology Unit, Cambridge, England. It has been shown to cross-react with other peptides of the PP family in gut endocrine cells, which seems due to minority antibody populations. Specificity controls were in the present work obtained by preincubating diluted antiserum at +4°C overnight with NPY, avian pancreatic polypeptide (APP), bovine pancreatic polypeptide (BPP) or peptide YY (PYY), all 100 μg/ml diluted antiserum. No staining was seen with antiserum that had been inactivated with NPY, whereas absorption with APP, BPP, and PYY did not influence appreciably the demonstrability of the NPY immunoreactivity in the tissue. APP, BPP, and PYY thus are not likely to be responsible for the NPY-like immunofluorescence seen in this work. Nevertheless, cross-reactions with unidentified peptides displaying the same antigenic site as NPY to the antibody cannot be excluded. Therefore, the structures revealed with the NPY antibody are referred to in this paper as NPY immunoreactive fibers or NPY immunoreactive neurons.

For comparison, the adrenergic fibers of the guinea-pig eye were demonstrated with the formaldehyde histofluorescence method of Falck and Hillarp. The animals used in this study have all been treated according to the ARVO Resolution on the Use of Animals in Research.

Results

Numerous NPY immunoreactive fibers were seen in all parts of the uvea. They were thin and had characteristic little oval beads (1-3 μm long, 1-2 μm across) at fairly regular intervals (about every 8-15 μm). The fluorescence intensity was often somewhat less in the intervaricose part than in the varicosity. In general, the uveal fibers seemed somewhat less immunoreactive than fibers around extraocular ciliary arteries or vessels occasionally encountered in extraocular muscles.

The appearance of the NPY immunoreactive fibers corresponds precisely to that seen in adrenergic peripheral neurons as demonstrated with the formaldehyde fluorescence method. In order to facilitate comparisons of the distribution of the adrenergic and NPY immunoreactive fibers, Figures 1-3 show the distribution of the noradrenergic fibers in the guinea-pig iris, ciliary processes, chamber angle, and retina. More detailed descriptions have been published. The results of the current reexamination corresponded well with the previous results.

Cornea and Sclera

The cornea and sclera were devoid of NPY immunoreactive fibers, also at the limbus and at the intrascleral collector vessels. A few NPY immuno-
reactive fibers could be seen around the episcleral vessels at the limbus.

The Chamber Angle

The meshwork of the chamber angle is known to contain many adrenergic fibers in guinea pigs, which is in contrast with most other animals. The adrenergic fibers are particularly common in the outermost part of this tissue (Fig. 2). In contrast, it contained only few, scattered NPY immunoreactive fibers, most in parts well away from the sclera (Fig. 4).

The Iris

NPY immunoreactive fibers were numerous in a narrow zone immediately in front of the dilator muscle (Fig. 5). Only rarely were any such fibers seen to penetrate into the muscle itself. They were less common in the other parts of the iris stroma, decreasing in numbers anteriorly. NPY immunoreactive fibers occurred in the adventitia of the major iridal vessels (Fig. 6) but not in numbers comparable with the adrenergic fibers. The NPY fibers did not seem to be associated with capillaries or other small vessels. Many of the immunoreactive stromal NPY fibers appeared to be associated with melanophores in the iris (Fig. 7), but some were without association with any identifiable structure.

Nerve fiber trunks in the iris stroma occasionally contained a small number of thin, varicose NPY immunoreactive fibers (Fig. 6).

The sphincter was supplied with a small number of essentially circumferentially running NPY immunoreactive fibers (Fig. 8). There was at times a tendency for the NPY immunoreactive fibers to be more numerous in the peripheral and posterior parts of the muscle. The immunoreactive fibers did not

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Fig. 2. Adrenergic fibers in the chamber angle of a normal albino guinea pig. Note the high number of fibers in the loose trabecular tissue near the sclera (asterisk). There is also a plexus of adrenergic varicose fibers at the pigment epithelium (PE). The picture is included in order to facilitate comparison with the NPY immunoreactive fibers in Fig. 4 (×140).

Fig. 3. Formaldehyde-induced fluorescence in guinea-pig retina, 4 hr after the intravitreal injection of 10 μg alpha-methyldopa mine. This treatment enhances the fluorescence of the dopaminergic neurons, which can be seen to form two sublayers of fibers in sublaminae 1 and 3 of the inner plexiform layer. There are also four dopaminergic cell bodies (arrows), one of which is signified out of focus. The picture is included to facilitate comparison with the NPY immunoreactive fibers in Figures 11 and 12. Ph: photoreceptors; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer (×175).
NPY immunoreactive fibers seem to be associated with the occasional fine vessels or melanophores in the muscle.

**The Ciliary Body and the Ciliary Processes**

NPY immunoreactive fibers were numerous in all ciliary processes (Figs. 5, 9, 10). They ran close to both blood vessels and the epithelium in the narrow stroma of the ciliary processes. No intraepithelial fibers were observed. No difference was found between anterior and posterior ciliary processes. There was a loose plexus of NPY immunoreactive fibers beneath the epithelium of the ciliary body all the way back to the ora serrata (Fig. 10).

The ciliary muscle contained a small-to-moderate number of mainly circumferentially and some radially directed delicate NPY immunoreactive fibers (Fig. 10). The number of such fibers did not appear dense enough to ascertain that every muscle cell is reached by a fiber. The number of fibers was roughly comparable with the number of adrenergic fibers.

**The Choroid**

The major arteries of the choroid were supplied with a well-developed plexus of NPY immunoreactive fibers, located at the border between the media and the adventitia, precisely coinciding with the position of the vascular adrenergic fibers (Fig. 11). However, the immunoreactivity was less than that in nerves around extraocular vessels. In addition, NPY immunoreactive fibers were common in the connective tissue around extraocular vessels.
Fig. 6. High-power micrograph of NPY immunoreactive fibers in the iris. Some occur in front of the dilator muscle (D), a few around a large vessel (V), and in the iris stroma. A few also appear in a nerve fiber bundle (NF). Above, fluorescence micrograph; below, phase contrast micrograph of the same region (×450).

tissue in between the choroidal vessels, running without any apparent connection with any identifiable structure. A few NPY immunoreactive fibers also were observed in nerve fiber bundles. Preterminal, nonvaricose immunoreactive fibers were not observed.

The Retina

Delicate, varicose NPY immunoreactive fibers were seen in a narrow sublayer of intertwined fibers in sublamina 1 of the inner plexiform layer, just at the border to the inner nuclear layer (Fig. 11). Very rarely, such fibers also could be seen in other sublayers of the inner plexiform layer. Immunoreactive cell bodies were seen in the innermost cell row of the inner nuclear layer. They were quite difficult to detect because their immunoreactivity often was concentrated to the hillock region with only very faint immunofluorescence in the rest of the perikaryon (Fig. 12). The nucleus was always devoid of immunofluorescence. The size of the cells was not estimated readily because of their weak immunoreactivity but was not distinctly different from the surrounding amacrine cells. The frequency of the cells was for the same reason difficult to estimate, but a judgement based on the number of hillock-like aggregations of immunoreactivity gave a figure of 5–10/mm section length. The NPY immunoreactive cells occurred at relatively regular intervals and were not seen in pairs.
or clusters. They were not obviously different in numbers in central or peripheral parts of the retina. Cell processes could be seen to reach the sublayer of NPY immunoreactive fibers in the inner plexiform layer. Several hundred sections have been examined, and an immunoreactive fiber was on two occasions seen to penetrate the inner nuclear layer to reach the outer plexiform layer. They were not seen to ramify there, and no immunoreactive fibers were detected in this layer. The NPY immunoreactive structures described were not connected with any retinal blood vessels.

Fig. 8. NPY immunoreactive fibers in the sphincter region of the iris. Note that there are a few such fibers scattered through the sphincter muscle (SP). Above, fluorescence micrograph; below, phase contrast micrograph of the same region (×180).
The Optic Nerve

The optic nerve was not found to contain any NPY immunoreactive fibers except an occasional perivascular twig.

Sympathetic Denervation

No NPY immunoreactive fibers remained in the ipsilateral anterior uvea 1 week after sympathetic denervation. However, the choroid and the most posterior quarter or fifth of the ciliary body showed a decreased but still significant number of NPY immunoreactive fibers after the operation (Fig. 13). The fibers were seen in all locations described above in the choroid, with a uniform decrease in their number. The immunoreactivity of the fibers seemed decreased.

No changes were seen in the NPY immunoreactive fiber density on the contralateral control side or in the retina on either side.

The Superior Cervical Ganglion

The superior cervical ganglion was included in the study in order to verify the origin of the NPY immunoreactive fibers disappearing on sympathetic denervation. It was seen to contain numerous cell bodies displaying varying, weak-to-moderate fluorescence intensities. The size and shape of the NPY immunoreactive neurons were not appreciably different from other neurons of the ganglion. There was no apparent connection between cell size and immunoreactivity. The NPY immunoreactive cells were scattered evenly throughout the ganglion. A few NPY immunoreactive fibers also were seen.
Discussion

Based on structural relationships, many neuropeptides can be grouped into families. NPY belongs to the family that also comprises the pancreatic polypeptides and peptide YY (PYY).\(^2\) Immunologic cross-reactivity therefore can be expected with these peptides and have, indeed, been revealed in gut endocrine cells with the antiserum used in this study\(^8\) as well as in studies on the brain.\(^3\) However, absorption of the antibody with these peptides did not diminish the immunoreactivity in the nerve fibers in the guinea-pig eye, which makes it likely that the material demonstrated is NPY rather than the pancreatic polypeptides or PYY.

In the retina, NPY was found in a subclass of
Fig. 13. NPY immunoreactive fibers around some large vessels in the choroid, 14 days after the excision of the homolateral cervical sympathetic ganglia. A significant number of immunoreactive fibers remain around the vessels and in the stroma, although the number is somewhat decreased compared with the normal. There is material with nonspecific fluorescence in the lumen of three vessels (v). (Fluorescence micrograph ×280).

amacrine cells. Several such subclasses are now known, different for various neuropeptides and different in various species (reviews10,11). However, the concentration of the NPY immunoreactivity to the hillock region of the cell body departs somewhat from what usually has been seen previously with other peptides, which distribute more or less evenly throughout the cytoplasm. The hillock region is where the Golgi complex is often found. Presumably then, NPY is accumulated into granules in the Golgi apparatus and then efficiently transported out into the processes, whereas only little is transported to the perikaryon. A similar accumulation of a regulatory peptide to the Golgi region previously has been noted only in some peripheral nerve cells.18

The NPY immunoreactive processes distribute almost exclusively to the outermost sublayer (sublamina 1) of the inner plexiform layer, which is the layer where many of the dopaminergic fibers are found in the guinea pig5,19 (Fig. 3). It therefore is worth noticing that NPY has been seen to coexist with a catecholamine in the human medulla oblongata.20 However, dopaminergic fibers also occur in sublamina 3 in the guinea-pig inner plexiform layer, which the NPY immunoreactive fibers do not. It therefore seems unlikely that NPY coexists with dopamine in the retina. Other neurotransmitters known or presumed in the guinea-pig retina have an even more different pattern of distribution.11

The distribution of the NPY immunoreactive fibers in the extraretinal parts of the eye is in many regions identical with the distribution of the noradrenergic sympathetic fibers originating in the superior cervical ganglion14–16,22 (see also Figs. 1, 2). The denervation experiments prove that the NPY immunoreactive fibers in the anterior uvea originate in the superior cervical sympathetic ganglion, and, confirming, the superior cervical ganglion was seen to contain numerous NPY immunoreactive cell bodies.

The similarity in fiber densities and distribution makes it likely that NPY is present in most noradrenergic fibers of the guinea-pig uvea. Lundberg et al.6 obtained direct evidence for such a coexistence in cats. They showed that the superior cervical ganglion in cats contains many NPY immunoreactive neurons, which also contain noradrenaline, as judged from their content of tyrosine hydroxylase and dopamine beta hydroxylase.

The observation that choroidal NPY immunoreactive fibers diminish in number but do not disappear upon ablation of the cervical sympathetic ganglion chain shows that in part they come from these ganglia, but in part also from somewhere else. The origin of the fibers remains to be determined.

The noradrenaline containing fibers in the guinea-pig uvea all originate in the superior cervical ganglion chain,14,15,21,22 and the NPY immunoreactive fibers that do not derive from this ganglion thus lack noradrenaline. Conceivably therefore, NPY ocular fibers can be divided into two classes, one where it coexists with noradrenaline, and one where it does not.

NPY immunoreactive fibers did not appear in certain places where noradrenergic ones can be found, most notably in the cornea and in parts of the chamber angle. They were also fewer than the adren-
neuroactive peptide and is directly comparable with the density of noradrenergic fibers. It therefore seems likely that NPY is present in most noradrenergic neurons in the eye. Since the sympathetic adrenergic nervous system participates in the regulation of the intraocular pressure and in the inflammatory responses in rabbits, the NPY immunoreactive cells were fewer than the adrenergic ones. They also noted that in several tissues in the cat, there were significantly fewer NPY immunoreactive fibers than adrenergic ones. The observations reinforce the conclusion that only certain noradrenergic neurons contain NPY.

NPY immunoreactive fibers occur around the arteries of the cat submandibular gland, and NPY has been shown to give a long-lasting vasoconstriction in this tissue, also after cervical sympatheticectomy. This suggests that there are NPY-agonistic, vasoconstrictive receptors, which are independent of sympathetic innervation. A different action also has been detected. NPY is present in nerve fibers in the mouse vas deferens. Here, it inhibited nerve-induced muscle contractions but had no effect when the organ was denervated sympathetically, suggesting that in this case, it acts to inhibit the release of noradrenaline from nerve terminals.

The relative paucity of NPY immunoreactive fibers around the iris blood vessels would suggest a minor role on vessels in this region. However, the choroidal vessels have a rich supply of NPY immunoreactive fibers, and the vasoconstrictive effects therefore may be more prominent in this part. It is worth noticing that these fibers originate only in part in the cervical sympathetic chain.

NPY has been associated previously with secretory cells in, eg, the salivary glands, the pancreas, and the gastrointestinal tract. It is, therefore, possible that the fibers seen in the ciliary processes affect the secretion of aqueous humour, and thus also the intraocular pressure.

The density of NPY immunoreactive fibers in the uvea is much higher than what has been found for any other neuroactive peptide and is directly comparable with the density of noradrenergic fibers. Since the sympathetic adrenergic nervous system participates in the regulation of the intraocular pressure and in the inflammatory responses in rabbits, the as yet unknown effects of NPY on these are of considerable interest.

Key words: neuropeptide Y, guinea pig, uvea retina

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