Cell Tissue Res (1987) 250:267–275

Cell and Tissue Research

Galanin-immunoreactive nerves in the rat iris: alterations induced by denervations

Ingrid Strömberg[1], Håkan Björklund[1], Tor Melander[1], Åke Rökaeus[2], Tomas Hökfelt[1], and Lars Olson[1]

[1] Department of Histology, Karolinska Institutet, Stockholm, Sweden;
[2] Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, USA

Summary. The iris and choroid membrane of the adult rat contain nerve fibers expressing immunoreactivity to the neuropeptide galanin. The density and distribution of galanin-positive nerve fibers varied from iris to iris and, particularly, among animals. Smooth, non-terminal axons were seen running in nerve bundles consisting of otherwise negative fibers. From the choroid membrane these bundles reached the iris via the ciliary body. Axons were frequently seen to branch giving rise to a sparse system of varicose, single fibers in the dilator plate and sphincter area. Galanin-positive fibers were sometimes also seen outlining blood vessels.

Capsaicin, in a dose that causes permanent depletion of substance P- and cholecystokinin-immunoreactive fibers in the iris, caused no change in amount of galanin-positive fibers. Removal of the superior cervical ganglion caused a rapid and pronounced increase in the number of galanin-immunoreactive nerve fibers. Similarly, removal of the ciliary ganglion appeared to increase galanin immunoreactivity, while removal of the pterygopalatine ganglion was less effective. Lesioning of the trigeminal ganglion caused a disappearance of galanin immunoreactivity. The sympathetectomy-induced increase was counteracted by capsaicin.

Galanin-positive nerve cell bodies were present in both the superior cervical and the trigeminal ganglia. In the superior cervical ganglion, immunoreactive galanin did not seem to coexist with neuropeptide Y-positive cells; in the trigeminal ganglion, some galanin-positive cells also contained calcitonin gene-related peptide (CGRP) immunoreactivity, while most cells did not. In the iris, double-staining suggested that CGRP and galanin immunoreactivities were contained in different fiber populations.

We conclude that the rat iris and choroid membrane contain a sparse plexus of nerve fibers expressing galanin-like immunoreactivity. It is suggested that these fibers are derived from the trigeminal ganglion. The iris is able to respond with a pronounced increase in number of galanin-immunoreactive nerve fibers to certain denervation procedures.

Key words: Galanin – Iris – Choroid membrane – Immunohistochemistry – Trigeminal ganglion – Superior cervical ganglion – Calcitonin gene-related peptide – Capsaicin – Rat

Send offprint requests to: Dr. Ingrid Strömberg, Department of Histology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Galanin is a 29 amino acid polypeptide found in porcine intestine (Tatemoto et al. 1983). It has been shown to express several biological effects; for example, it is able to contract intestinal smooth muscle preparations and cause a hyperglycemic reaction in dogs due to inhibition of insulin release (Tatemoto et al. 1983; see Rökaeus 1987). Recently, antisera against galanin were developed (Rökaeus et al. 1984b) and galanin-like immunoreactivity was found in widespread unique systems of the central nervous system, gastrointestinal tract and pancreas as well as in the adrenal medulla and urogenital tract (Rökaeus et al. 1984a; Ch'ng et al. 1985; Ekblad et al. 1985b; Skofitsch and Jacobowitz 1985a, b; Melander et al. 1985a, b, 1986a, b; Bauer et al. 1986; Dunning et al. 1986; Rökaeus 1987). In several cases, galanin-like immunoreactivity seems to coexist with classical neurotransmitters and/or other neuropeptides (Rökaeus et al. 1984a; Melander et al. 1985b, 1986b).

The complex innervation of the mammalian iris (e.g., Tervo et al. 1982) has recently been reviewed in detail (Olson et al. 1987). Sympathetic and parasympathetic nerves originating in the superior cervical and ciliary ganglia, respectively (cf. Ehinger and Falck 1966), form dense networks in the iris. The trigeminal ganglion supplies the iris with a considerable number of both thick, myelinated and thinner, unmyelinated fibers, which can be observed using silver-staining techniques (Ayer-LeLievre et al. 1984) or immunohistochemistry using antisera against the neurofilament triplet (Seiger et al. 1984). Recently, several peptide-containing nerve fiber networks have been identified in the iris. Thus, substance P-positive varicose fibers have been shown to form a relatively dense plexus (Hökfelt et al. 1977; Cuello et al. 1978; Miller et al. 1981; Seiger et al. 1985). They are capsaicin-sensitive and originate from cell bodies in the trigeminal ganglion. We have earlier demonstrated the presence of enkephalin- (Björklund et al. 1984), vasoactive intestinal polypeptide- and cholecystokinin- (Björklund et al. 1985a) immunoreactive nerves in the rat iris. Recently, calcitonin gene-related peptide (CGRP)-positive nerves originating in the trigeminal ganglion were also described (Terenghi et al. 1985; Olson et al. 1987). Whereas the cholecystokinin-, substance P- and CGRP-positive network disappears after capsaicin treatment and probably originates in the trigeminal ganglion, the enkephalin- and vasoactive intestinal polypeptide-positive nerve fibers do not have their origin in any of the three ganglia with known projections to the iris. Similarly, Uddman et al. (1980) have reported the presence of vasoactive intestinal polypeptide-positive

nerve fibers in the choroid membrane of the rat. These investigators also demonstrated that the vasoactive intestinal peptide-positive fibers originated in the pterygopalatine ganglion.

In the present study we have investigated the distribution of galanin-positive nerve fibers in the normal iris and choroid membrane. Furthermore, we have attempted to elucidate the origin of these fibers by selective denervations and double-staining procedures.

Materials and methods

Animals and denervation procedures

Adult albino rats (Sprague-Dawley) of both sexes were used. The presence and distribution of galanin-immunoreactive fibers were studied in normal irides and irides that had been denervated by removal of the superior cervical ganglion, the ciliary ganglion or the pterygopalatine ganglion, or by lesioning the trigeminal ganglion or cutting the nasociliary nerve. All operations were performed under ether anesthesia except trigeminal lesions, where Halothane anesthesia was used. Superior cervical ganglionectomies were made bilaterally, while all other denervations were made unilaterally. Sympathetically-denervated rats were sacrificed 1, 2, 4 and 6 days, 1 month and 2.3 years after denervation. Pterygopalatinectomized, ciliarectomized, and nasociliary nerve-transected rats were sacrificed 6 days postoperatively, while animals with lesions of the trigeminal ganglion were sacrificed 7 days postoperatively. Capsaicin, 50 mg/kg, dissolved in a mixture of 10% ethanol and 10% Tween 80 in NaCl, was injected subcutaneously in normal and sympathetically denervated rats. They were examined 3 days after injection. Control animals were injected with vehicle alone.

Colchicine injections were made stereotaxically to the trigeminal ganglion under Halothane anesthesia (coordinates: 4 mm caudal, and 3 mm lateral to bregma, 10.4 mm below the dura). Colchicine was dissolved in Ringer's solution in a concentration of 6μg/μl and 5μl were injected over 2 min two days prior to sacrifice. The superior cervical ganglion was locally treated with colchicine by instillation of gelfoam soaked with colchicine close to the ganglion.

Immunohistochemistry

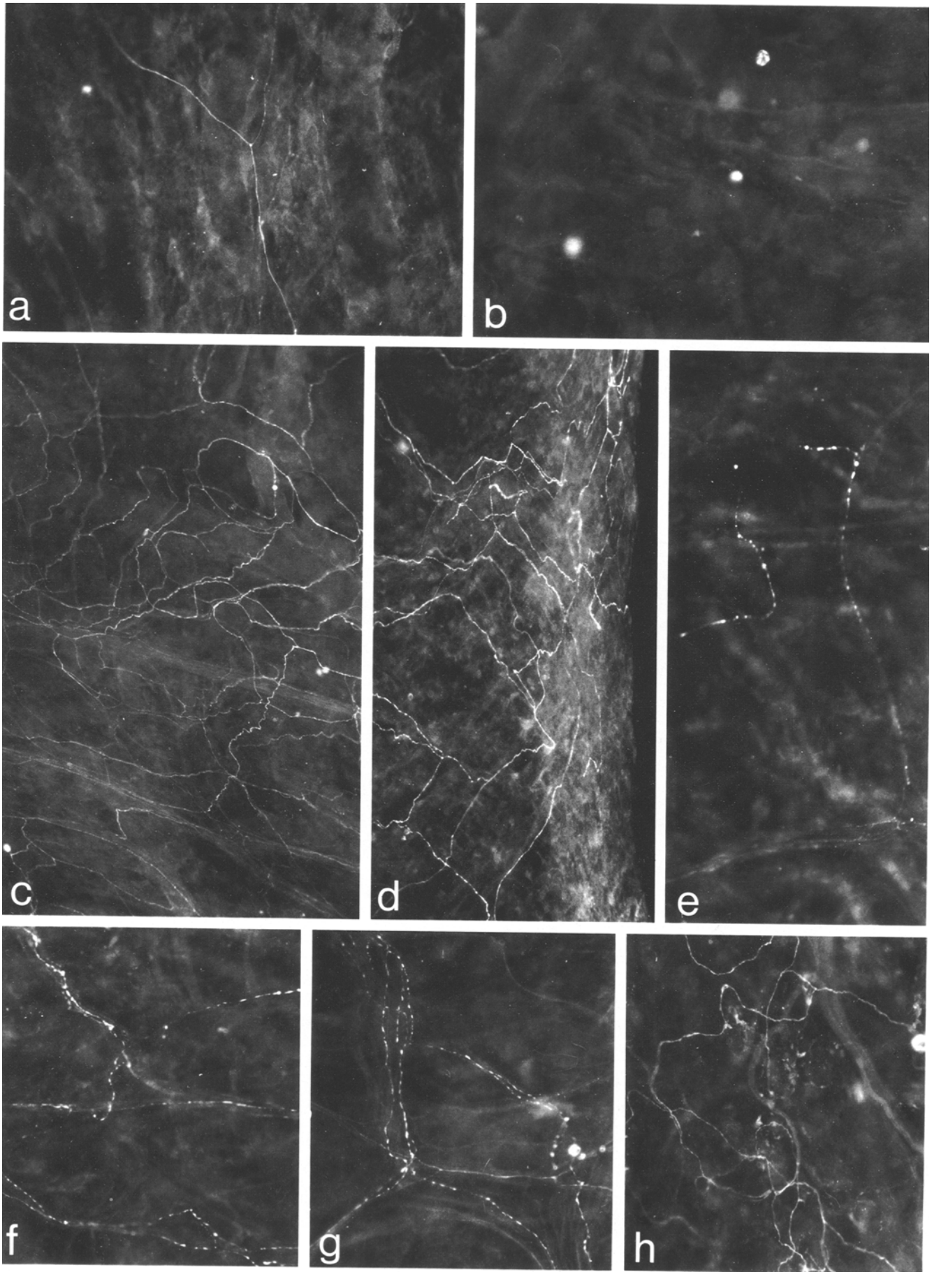
Normal, denervated and capsaicin-treated animals were sacrificed by exsanguination under deep ether anesthesia. Irides were prepared and fixed mainly according to the method of Costa et al. (1980) as modified for this material by Ayer-LeLievre and Seiger (unpublished): they were gently stretched, pinned to a plastic surface with very fine needles and covered with a picric acid/formaldehyde mixture (Stefanini et al. 1967). They were then rinsed in phosphate-buffered saline (PBS).

The indirect immunofluorescence technique (see Coons 1958) was applied to floating irides. The characteristics of the pig galanin antiserum (R2-8310) produced in rabbit have been described elsewhere (Rökaeus et al. 1984a). Most of the experiments were, however, carried out with a galanin antiserum purchased from Peninsula (Belmont, Calif., USA). Both antisera were diluted 1:400. Control irides were incubated in antiserum absorbed with an excess of synthetic galanin (Peninsula). For double-staining, irides were incubated in a mixture of galanin antiserum (1:400) and CGRP antiserum raised in goat (Tschopp et al. 1984) (1:400). Irides were incubated with antisera overnight at 4° C, rinsed 3 × 10 min in PBS and incubated with fluorescein isothiocyanate- (FITC) conjugated anti-rabbit antibodies (1:40; Boehringer Mannheim Scandinavia, Stockholm, Sweden) or for the double-stained irides, with a mixture of donkey anti-goat FITC-conjugated antibodies (1:10; Nordic Immunological Laboratories, Tilburg, The Netherlands) and swine anti-rabbit tetramethyl rhodamine isomer R (TRITC, rhodamine) (1:10; Dakopatts, Copenhagen, Denmark) for 1 h at room temperature, rinsed in PBS, mounted in a mixture of glycerol and phosphate buffer (9:1) containing 0.1% p-phenylene diamine as an antifading agent (Johnson and C. de Nogueira Araujo 1981; Platt and Michael 1983), and examined in dark-field fluorescence microscopes equipped with the appropriate filter combinations. Although specificities of the antisera have been tested, it should be kept in mind that immunohistochemistry does not permit molecular identification of antigens. Thus, the term galanin-like immunoreactivity is appropriate to describe the level of precision of the technique.

To ascertain that the sympathetic denervation was complete and permanent in animals denervated 2.3 years before sacrifice, a small sector from each such iris was processed for histochemical visualization of catecholamines (Falck 1962; Falck et al. 1962).

For studies of the superior cervical and trigeminal ganglion, animals were deeply anesthesized with pentobarbital and fixed by perfusion through the aorta with Tyrode's solution followed by a paraformaldehyde-picric acid mixture (~0.4% picric acid and 10% formalin in 0.2 M sodium phosphate buffer, pH 6.9) for 6 min. Brains were then postfixed by immersion in the same fixative for 90 min, rinsed in phosphate buffer with 10% sucrose and 0.02% Bacitracin (Bayer, Leverkusen, FRG) and sodium azide (0.1%) for at least 24 h. Cryostat sections, 14 μm thick, were collected onto chromalun gelatin-coated slides. Sections were then treated with antisera using indirect immunfluorescence as described above. Elution of the galanin antibody and res-

Fig. 1a–h. Appearance of nerve fibers with galanin-like immunoreactivity in whole-mount preparations of rat irides under normal and experimental conditions. a, b, e, f, g and h ×300; c and d ×125. Dark-field microphotographs. **a** Normal iris. A few partly varicose fibers are seen in the dilator plate. **b** One week after lesioning of the trigeminal ganglion, no galanin-positive fibers can be observed. **c, d** Increase in galanin-immunoreactive fibers following removal of the superior cervical ganglion. **c** A rich network in the dilator plate 6 days after sympathectomy. In **d**, nerve fibers are seen in the sphincter region (right) and neighboring areas of the dilator plate one month after sympathetic denervation. **e** Varicose, scattered galanin-positive fibers seen two years and three months after removal of the superior cervical ganglion. At this time, the density does not differ from normal. **f** Transection of the nasociliary nerve causes a slight increase in galanin-positive nerve fibers in the dilator plate. **g** Removal of the pterygopalatine ganglion causes a moderate increase of galanin-immunoreactive nerve fibers. **h** Removal of the ciliary ganglion causes a clear-cut increase in number of galanin-immunoreactive nerve fibers. The relative proportion of smooth and varicose fibers may differ from area to area and from iris to iris


a
b
c
d
e
f
g
h

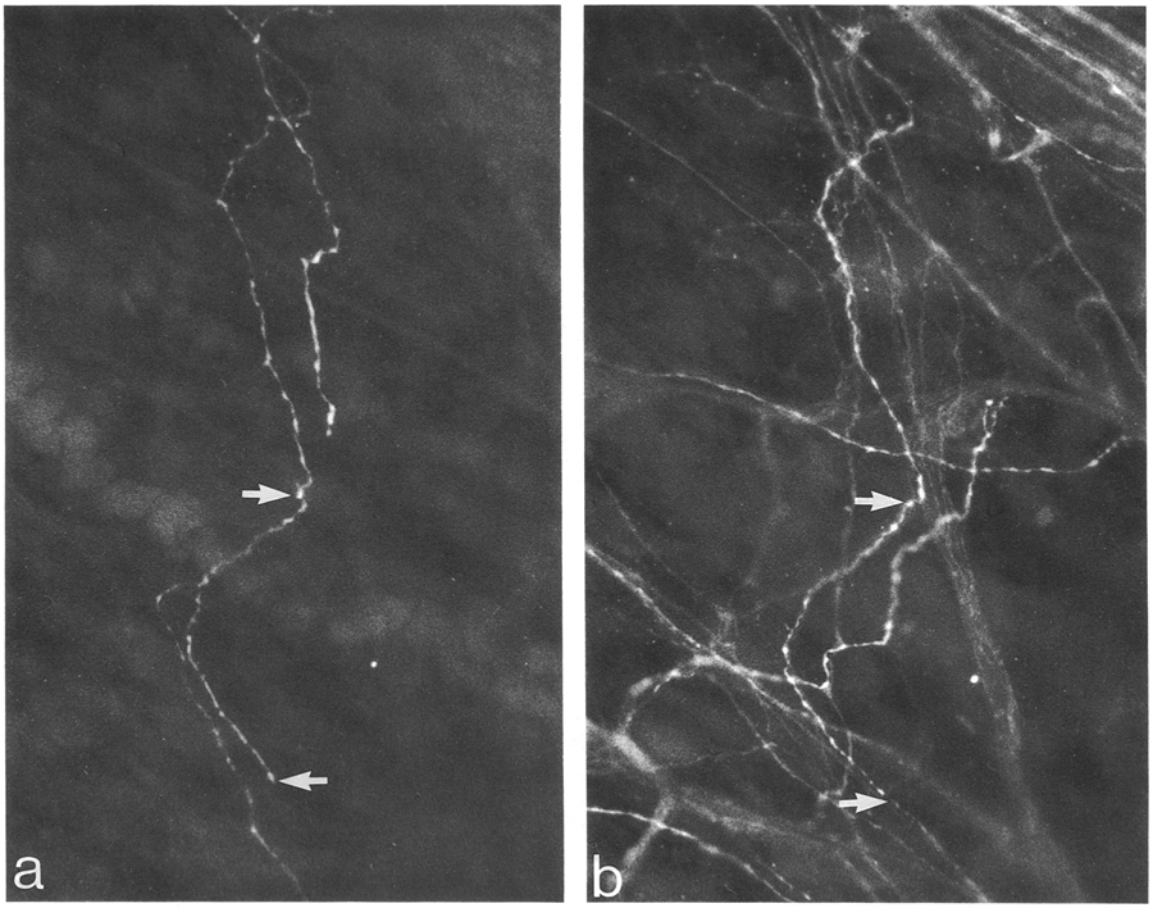


Fig. 2a, b. Double-staining of a rat iris for galanin- and CGRP-like immunoreactivities using a mixture of primary antisera raised in different species and secondary antibodies labeled with different fluorophores. Galanin-positive fibers are depicted in **a**, CGRP-positive fibers in **b**. At close inspection, galanin-positive fibers can be seen to run for long distances together with CGRP-positive fibers. However, the distribution of varicosities along the fibers differs, and there are also examples of galanin fibers that do not run together with CGRP fibers and vice versa. *Arrows* in the two figures point to some of the differences. Fluorescence microphotographs, ×450

taining with an antiserum against neuropeptide Y (1:400; Lundberg et al. 1984) in sections of the superior cervical ganglia were carried out as described by Tramu et al. (1978). Briefly, coverslips were removed and slides rinsed and dipped into a solution of potassium permanganate for 50 s, rinsed, incubated with FITC-conjugated antibodies, analyzed in the fluorescence microscope and, if no fluorescence was observed, rinsed again and restained with NPY antiserum. Double-staining of sections of the trigeminal ganglion for galanin and CGRP employed mixtures of two primary antisera raised in different species, as described above for the irides.

Results

Normal irides

Whole-mount preparations of irides included portions of the choroid membrane, the ciliary body and its processes, as well as the dilator plate and sphincter margin of the iris. Almost all normal irides contained an irregular, sparse to very sparse network of galanin-positive varicose fibers (Fig. 1a). The distribution and number of fluorescent fibers were, however, surprisingly variable from iris to iris, and in particular, from rat to rat, so that the amount of nerves was about the same in the right and left eyes, and thus occasional irides only contained a few fluorescent fibers. Galanin-positive fibers entered the iris from the choroid membrane via the ciliary body in thick nerve bundles consisting of otherwise negative nerve fibers. From such relatively smooth fibers, branching of more varicose fibers into the dilator plate was observed. Even within irides an uneven distribution was noted so that some areas were sparsely innervated, usually close to the ciliary body, whereas other portions lacked galanin-positive fibers. Sometimes galanin-immunoreactive fibers were seen associated with blood vessels of the dilator plate. Fibers were also found in the sphincter area. A comparatively high number of fluorescent fibers was observed forming an irregular network in the choroid membrane. Also in this tissue individual fluorescent fibers could be seen running in otherwise negative fiber bundles.

The possible coexistence of galanin and CGRP in nerve fibers of the iris was tested in normal irides double-stained to visualize antibodies against both peptides. Although no such coexistence could be demonstrated, the low number of galanin-positive fibers made comparisons difficult. Double-staining was therefore also performed in a group of irides that had been sympathetically denervated one month earlier to increase the network of galanin-immunoreactive fibers (see below). However, also in this case, we were unable to demonstrate clear cases of coexistence, although

Table 1. Effects of various denervation procedures on the number of galanin-immunoreactive nerve fibers in the rat iris. The density of the galanin-positive network was estimated on coded slides using a 0–5 scale, where 5 represents the density of the sympathetic ground plexus (well-known standard)

Denervation	Nerve amount (semiquantitive estimations)	*n*
Normal irides	0.75	23
Superior cervical ganglionectomy	3.7	30
Ciliary ganglionectomy	3.5	2
Pterygopalatine ganglionectomy	2.5	2
Nasociliary nerve transection	1.0	2
Lesion of trigeminal ganglion	0	2
Capsaicin 50 mg/kg	0.75	8
Superior cervical ganglionectomy + capsaicin	0.75	8

sometimes galanin- and CGRP-immunoreactive fibers ran close together within the same nerve fiber bundle, possibly within the same Schwann cell ensheathment, for considerable distances before one of them departed, revealing that they were actually separate fibers running close together. This is illustrated in Fig. 2a, b.

Effects of denervations

As summarized in Table 1 three types of denervations – removal of either the superior cervical, the ciliary or the pterygopalatine ganglion – all caused pronounced increases in the number of galanin-immunoreactive nerve fibers. This interesting reaction was most thoroughly studied in the case of superior cervical ganglionectomies. There was an increase in number of visible fibers and, to some extent, in fluorescence intensity of individual fibers. The pattern remained irregular with areas almost totally devoid of fibers and other areas of the iris with relatively rich networks. There was a slight increase in both fluorescence intensity of individual fibers and number of visible fibers already one day after sympathetic denervation; these increases were more pronounced after two days, and even more marked after one month. In a group of animals sacrificed two years and three months after sympathectomy, galanin immunoreactivity did not differ from normal. A small portion of these irides was processed for Falck-Hillarp fluorescence histochemistry: the irides were still totally devoid of noradrenergic catecholamine-containing fibers. The increase in galanin-immunoreactive nerve fibers seen 6 days and 1 month after sympathetic denervation is illustrated in Fig. 1c and d; the return to normal density 2.3 years after denervation in Fig. 1e. As indicated in Table 1, transection of the nasociliary nerve caused a slight increase in galanin-immunoreactive nerve fibers (Fig. 1f), while removal of the ciliary or the pterygopalatine ganglion caused more clear-cut increases (Fig. 1g and h, respectively).

A stereotaxic electrothermal lesion of the trigeminal ganglion caused a complete disappearance of nerve fibers with galanin-like immunoreactivity in the iris, as summarized in Table 1 and illustrated in Fig. 1b.

Capsaicin in a dose that is known to cause depletion of the intrinsic substance P network in the iris, caused no disappearance of galanin-positive fibers, either in the iris or in the choroid membrane. However, it seemed as if capsaicin given to previously sympathectomized animals caused a disappearance of the sympathectomy-induced increase in galanin immunoreactivity.

The superior cervical ganglion

Galanin-positive cell bodies were distributed throughout the colchicine-treated ganglion along with strongly fluorescent fibers and varicosities (Fig. 3a, c). The varicosities were very often seen in direct contact with both labeled and negative neuronal somata. Immunoreactive cell bodies were often clustered together in small groups (Fig. 3a).

Elution of the galanin antibody and restaining with an antibody against neuropeptide Y revealed that most of the galanin-positive cells were neuropeptide Y-negative (Fig. 3a–d), thus indicating that galanin and neuropeptide Y-positive cells in the superior cervical ganglion are largely separate neuronal populations.

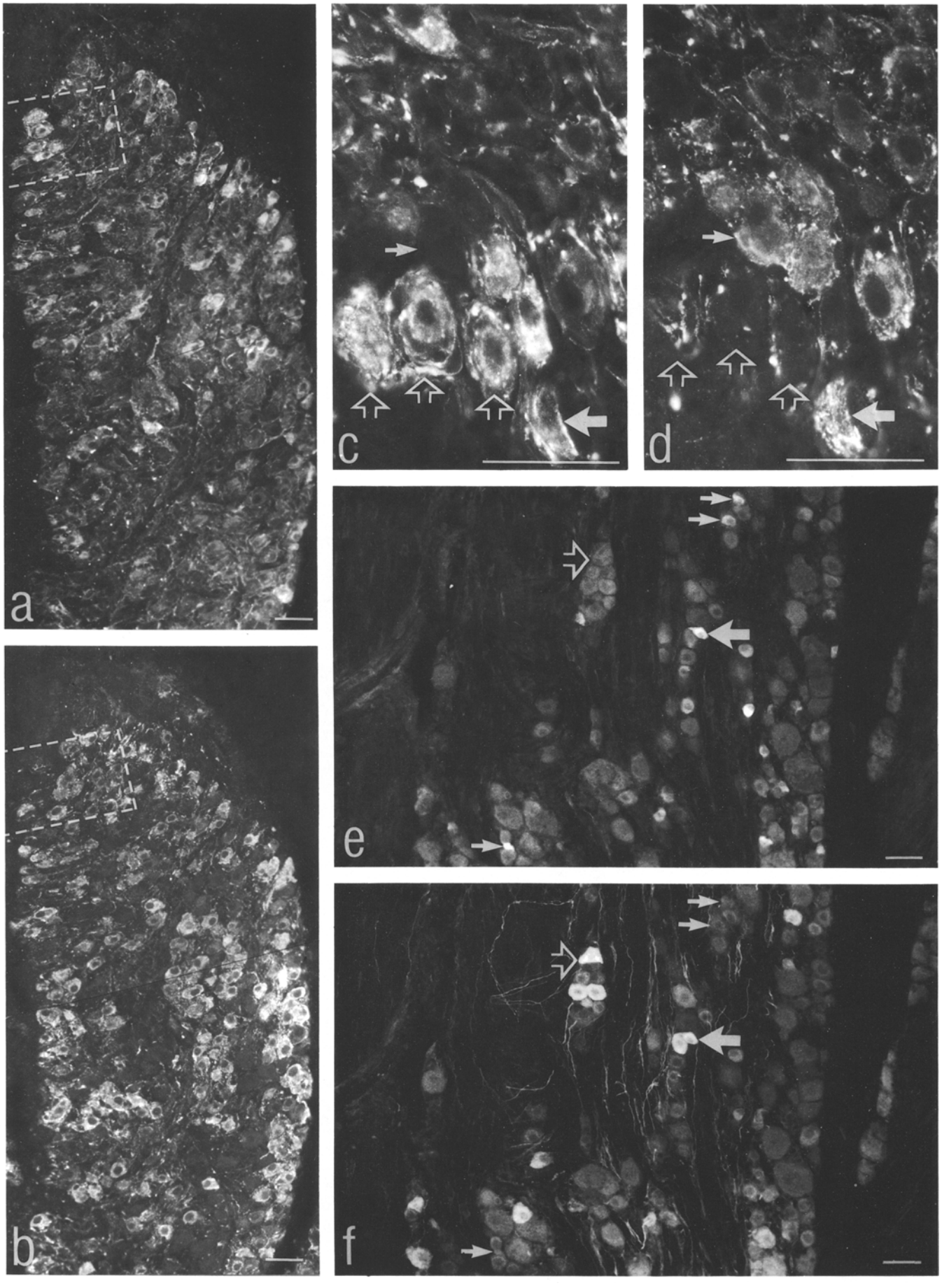
The trigeminal ganglion

Galanin-positive cell bodies were seen rather sparsely distributed throughout the trigeminal ganglion (Fig. 3e). They formed a population of small cell bodies, usually individually distributed as opposed to the clusters seen in the superior cervical ganglion. Double-staining using a mixture of antibodies to CGRP and galanin revealed a small population of cells that contained both galanin- and CGRP-like immunoreactivity. However, a proportion of the galanin-positive cells was CGRP-negative, and many CGRP-positive cells were galanin-negative. Very few galanin-immunoreactive nerve fibers were seen, whereas a large number of CGRP-positive fibers could be detected.

Discussion

The present study demonstrates a sparse, irregular system of nerve fibers expressing galanin-like immunoreactivity in the iris and the choroid membrane of adult rats. Galanin-like immunoreactivity has earlier been observed in widespread systems of the central nervous system (see Introduction). In the peripheral nervous system galanin-positive neuronal structures have so far been demonstrated in large numbers in the enteric nervous system (Ekblad et al. 1985b; Melander et al. 1985a) and in sensory neurons (Skofitsch and Jacobowitz 1985b; Ju et al. 1987). Our findings of galanin-immunoreactive nerve fibers in the anterior uvea indicates that these neuropeptides might be distributed also in other peripheral systems (see below).

The innervation apparatus of the rat iris has recently been reviewed (Olson et al. 1987). A large number of different neuropeptides have been observed immunohistochemically in the anterior uvea of the rat; these include substance


a
b
c
d
e
f

P (Hökfelt et al. 1977; Cuello et al. 1978; Miller et al. 1981; Tervo et al. 1981; Seiger et al. 1985), enkephalin (Björklund et al. 1983, 1984), neuropeptide Y (Terenghi et al. 1982; Björklund et al. 1985b), vasoactive intestinal polypeptide (Björklund et al. 1985a), CGRP (Terenghi et al. 1985; Olson et al. 1987), and cholecystokinin (Björklund et al. 1985a), although recent evidence indicates that CCK-like immunoreactivity in sensory neurons may represent cross-reactivity with CGRP or a similar peptide (Ju et al. 1986). It is thus evident that the innervation of the mammalian anterior uvea is complex. The finding of a network of galanin-immunoreactive nerve fibers in the iris adds to this complexity.

Galanin-positive nerve cell bodies were found both in the superior cervical and the trigeminal ganglia. However, removal of the superior cervical ganglion did not cause any disappearance of galanin-positive nerve fibers in the iris. On the contrary, a marked increase in amount of galanin-immunoreactive fibers was noted. This suggests, although it does not prove, that the galanin-positive nerve fibers seen in normal irides do not arise from the superior cervical ganglion. The superior cervical ganglionectomies do prove, however, that the increased number of galanin-positive fibers does not originate from this ganglion. Similarly, removal of the ciliary or the pterygopalatine ganglion increased the number of galanin-positive fibers, suggesting that their origin should be sought outside these ganglia as well. A stereotaxic lesion of the trigeminal ganglion 7 days prior to sacrifice completely abolished galanin-positive fibers in the iris. This strongly suggests a trigeminal origin for the galanin innervation.

Unlike two other trigeminal-derived neuropeptide-containing systems in the iris, substance P (see Seiger et al. 1985) and cholecystokinin (Björklund et al. 1985a), the normally present galanin-immunoreactive fibers do not appear to be affected by capsaicin treatment.

The increases in numbers of galanin-immunoreactive nerve fibers in the iris seen after several different types of denervations such as removal of the superior cervical, the ciliary or the pterygopalatine ganglion, or transection of the nasociliary nerve suggest that detectable levels of galanin-like immunoreactivity in the iris can change rapidly in response to changes in the innervation status of the iris. Similarly, we have shown earlier that enkephalin-like immunoreactivity is increased in the eye by denervation procedures (Björklund et al. 1984). Moreover, neuropeptide Y- as well as the noradrenergic marker tyrosine hydroxylase-like immunoreactivities can appear in what seems to be cholinergic nerves in the iris following sympathectomy (Björklund et al. 1985b). Taken together, these observations suggest interesting interrelationships between the various sets of nerves innervating the iris in which removal of certain nerves causes immediate appearance or increase of neuroactive peptides in other remaining nerves, suggesting that some of those neurons may change their phenotypical expression. Thus, the levels of peptides in the various compartments of the iris innervation apparatus seem to be in a dynamic equilibrium.

Further support for labile and perhaps readily altered levels of galanin-like immunoreactivity in the irides comes from the fact that there was a pronounced interindividual variation, such that the two irides of a given individual could contain the same amount of nerve fibers, whereas the two irides of another animal in the same experiment sometimes contained a very different number of nerves. Somewhat surprisingly, the distribution of galanin-immunoreactive nerve fibers in an individual normal iris was also found to be very variable, with some areas devoid of fibers, whereas other areas were innervated. The situation is similar to what has been found with vasoactive intestinal polypeptide-like immunoreactivity (Björklund et al. 1985a). Whether these variations are due to technical difficulties, uneven concentrations of the antigens within larger nerve networks, or represent true differences in densities of galanin and vasoactive intestinal polypeptide-positive nerves within the iris remains to be determined.

Although longstanding (one month), the increase in galanin immunoreactivity seen after sympathetic denervation is probably not permanent, since it was not seen 2 years and 3 months after denervation. However, this return to seemingly normal levels of galanin might also have been influenced by aging of the experimental animals.

To elucidate further the nature and origin of galanin-immunoreactive nerves in the iris, double-staining procedures were used. In the superior cervical ganglion, most of the galanin-positive cell bodies were separate from the neuropeptide Y-positive cells. This does not exclude that some of the galanin-positive nerve fibers in the normal iris are derived from the superior cervical ganglion. In the trigeminal ganglion, the most likely source of the galanin-positive nerves in the iris, the majority of the galanin-immunoreactive cell bodies were CGRP-negative, although a small population contained both CGRP and galanin-like immunoreactivity. In the iris, careful observations of the course and morphology of individual varicose nerve fibers double-stained for CGRP and galanin suggested that these two peptides were mostly located in separate nerve fibers. This was most easily seen after sympathetic denervation when the number of galanin-immunoreactive nerve fibers had increased. It should be noted, however, that also in this situation the number of galanin-immunoreactive nerve fibers was considerably smaller than the number of CGRP-immunoreactive fibers.

The function of galanin in the iris under normal circumstances and after perturbations of the innervation apparatus remains to be established. In the rabbit iris addition of

Fig. 3a–f. Immunofluorescence microphotographs of superior cervical (**a–d**) and trigeminal (**e, f**) ganglia after incubation with galanin antiserum (a, c, e), neuropeptide-Y antiserum (b, d) and CGRP-antiserum (f). **a** Numerous galanin-positive cell bodies are seen in the superior cervical ganglion, often forming clusters of positive somata. **b** Same section as in **a** after restaining for neuropeptide Y to illustrate the different distributions of the two peptides. In **c** and **d**, the *boxed areas* of **a** and **b** have been enlarged to demonstrate galanin-positive neuropeptide Y-negative (*hollow arrows*), as well as neuropeptide Y-positive/galanin-negative (*small filled arrow*) somata and one cell probably containing both immunoreactivities (*large filled arrow*). In **e** and **f** the same section was double-stained with antisera against galanin and CGRP respectively. Several galanin-positive/CGRP-negative (*small filled arrows*) and CGRP-positive/galanin-negative (*hollow arrow*) somata are seen. In addition, a small number of galanin/CGRP co-containing cell profiles can be detected (*large filled arrow*). a, b, c, and f ×118; c and d ×330

galanin has been shown to reduce the acetylcholine-mediated, but not the substance P-mediated contraction evoked by electrical stimulation (Ekblad et al. 1985a). These authors suggested a neuromodulatory effect at a presynaptic level. Similarly, a presynaptic modulatory effect of galanin has been demonstrated in the isolated vas deferens (Ohhashi and Jacobowitz 1985), in central tissues on dopamine release from fragments of the median eminence in vitro (Nordström et al. 1987), and on the isolated newborn rat spinal cord preparation, where galanin exerts a depressant effect on motoneurons (Yanagisawa et al. 1986).

In conclusion, a system of nerve fibers expressing galanin-like immunoreactivity has been described in the rat iris. They probably arise in the trigeminal ganglion. Normally, this system can be seen as scattered fibers, but following several different iris denervation procedures, the number markedly increases, possibly reflecting increases in peptide levels. These observations add yet another facet to the complexity of the innervation apparatus of the iris and the dynamic equilibrium between the expression of its different neuropeptides (see Olson et al. 1987).

Acknowledgements. Supported by the Swedish Medical Research Council (14X-03185, 04X-2887, 14R-6944, 14F-6944, 14F-7652), Magnus Bergvalls Stiftelse, Karolinska Institutet, Knut and Alice Wallenbergs Stiftelse, Hans and Loo Ostermans Foundation, the "Expressen" Prenatal Research Fund, and Åke Wibergs Foundation. We thank Karin Lundströmer, Carina Ohlsson, and Barbro Standwerth for skillful technical assistance and Ida Engqvist for expert secretarial help. We thank Prof. L. Terenius, Uppsala University, Sweden, and Prof. J. Fisher, Zürich University, Switzerland, for generous supplies of NPY and CGRP antisera, respectively.

References

Ayer-LeLievre CS, Granholm A-Ch, Seiger Å (1984) Silver impregnation of intrinsic sensory innervation of rat iris in toto. J Anat 138:309–321

Bauer FE, Christofides ND, Hacker GW, Blank MA, Polak JM, Bloom SR (1986) Distribution of galanin immunoreactivity in the genitourinary tract of man and rat. Peptides 7:5–10

Björklund H, Olson L, Seiger Å (1983) Enkephalin-like immunoflourescence in nerves of the rat iris following systemic capsaicin injections. Med Biol 61:280–282

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

Björklund H, Fahrenkrug J, Seiger Å, Vanderhaeghen J-J, Olson L (1985a) On the origin and distribution of vasoactive intestinal polypeptide-, peptide PHI- and cholecystokinin-like-immunoreactive nerve fibers in the rat iris. Cell Tissue Res 242:1–7

Björklund H, Hökfelt T, Goldstein M, Terenius L, Olson L (1985b) Appearance of the noradrenergic markers tyrosine hydroxylase and neuropeptide Y in cholinergic nerves of the iris following sympathectomy. J Neurosci 5:1633–1643

Ch'ng JLC, Christofides ND, Anand P, Gibson SJ, Allen YS, Su HC, Tatemoto K, Morrison JFB, Polak JM, Bloom SR (1985) Distribution of galanin immunoreactivity in the central nervous system and the responses of galanin-containing neuronal pathways to injury. Neuroscience 16:343–354

Coons AH (1958) Fluorescent antibody methods. In: Danielli JF (ed) General Cytochemical Methods, vol. 1, Academic Press, New York, pp 399–422

Costa N, Buffa R, Furness JB, Solcia E (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole-mount preparations. Histochemistry 65:156–165

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

Dunning BE, Ahren B, Veith RC, Böttcher G, Sundler F, Taborsky Jr GJ (1986) Galanin: a novel pancreatic neuropeptide. Am J Physiol 251:E127–E133

Ehinger B, Falck B (1966) Concomitant adrenergic and parasympathetic fibers in the iris. Acta Physiol Scand 67:201–207

Ekblad E, Håkanson R, Sundler F, Wahlestedt C (1985a) Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. Br J Pharmacol 86:241–246

Ekblad E, Rökaeus Å, Håkanson R, Sundler S (1985b). Galanin nerve fibers in the rat gut: Distribution, origin and projections. Neuroscience 16:355–363

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

Falck B, Hillarp N-Å, Thieme G, Torp A (1962) Fluorescence of catecholamines and related compounds condensed with formaldehyde. J Histochem Cytochem 10:348–354

Hökfelt T, Johansson O, Kellerth J-O, Ljungdahl Å, Nilsson G, Nygårds A, Pernow B (1977) Immunohistochemical distribution of substance P. In: von Euler US, Pernow B (eds) Substance P. Nobel Symposium 37. Raven Press, New York, pp 117–145

Johnson DG, de C Nogueira Araujo GM (1981) A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 43:349

Ju G, Hökfelt T, Fischer JA, Frey P, Rehfeld JF, Dockray GJ (1986) Does cholecystokinin-like immunoreactivity in rat primary sensory neurons represent calcitonin gene-related peptide? Neurosci Lett 68:305–310

Ju G, Hökfelt T, Brodin E, Fahrenkrug J, Fischer JA, Frey P, Elde RP, Brown JC (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide (CGRP) immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. Cell Tissue Res 247:417–431

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

Melander T, Hökfelt T, Rökaeus Å, Fahrenkrug J, Tatemoto K, Mutt V (1985a) Distribution of galanin-like immunoreactivity in the gastrointestinal tract of several mammalian species. Cell Tissue Res 239:253–270

Melander T, Staines WA, Hökfelt T, Rökaeus Å, Eckenstein F, Salvaterra PM, Wainer BH (1985b) Galanin-like immunoreactivity in cholinergic neurons of the septum-basal forebrain complex projecting to the hippocampus of the rat. Brain Res 360:130–138

Melander T, Hökfelt T, Rökaeus Å (1986a) Distribution of galanin-like immunoreactivity in the rat central nervous system. J Comp Neurol 248:475–517

Melander T, Hökfelt T, Rökaeus Å, Cuello AC, Oertel W, Verhofstad A, Goldstein M (1986b) Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. J Neurosci 6:3640–3654

Miller A, Costa M, Furness JB, Chubb IW (1981) Substance P immunoreactive sensory nerves supply the rat iris and cornea. Neurosci Lett 23:243–249

Nordström Ö, Melander T, Hökfelt T, Bartfai T, Goldstein M (1987) Evidence for an inhibitory effect of the peptide galanin on dopamine release from the median eminence. Neurosci Lett 73:21–26

Ohhashi T, Jacobowitz DM (1985) Galanin potentiates electrical stimulation and exogenous norepinephrine-induced contractions in the rat vas deferens. Regul Pept 12:163–171

Olson L, Ayer-LeLievre C, Björklund H, Ebendal T, Granholm AC, Hedlund K-O, Hökfelt T, Melander T, Seiger Å, Ström-

berg I (1987) The innervation apparatus of the rodent iris. In: Owman Ch, Björklund A, Hökfelt T (eds) Handbook of Chemical Neuroanatomy, Vol. 6, Elsevier, Amsterdam (in press)

Platt JL, Michael AF (1983) Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. J Histochem Cytochem 31:840–842

Rökaeus Å (1987) Galanin – a new isolated biologically active neuropeptide. TINS 10:158–164

Rökaeus Å, Melander T, Hökfelt T, Lundberg JM, Tatemoto K, Carlquist M, Mutt V (1984a) A galanin-like peptide in the central nervous system and intestine of the rat. Neurosci Lett 47:161–166

Rökaeus Å, Tatemoto K, Carlquist M, Mutt V (1984b) Galanin – a newly isolated peptide. Radioimmunoassay, distribution and effects. Dig Dis Sci 29:715

Seiger Å, Dahl D, Ayer-LeLievre Ch, Björklund H (1984) Appearance and distribution of neurofilament immunoreactivity in iris nerves. J Comp Neurol 223:457–470

Seiger Å, Selin UB, Kessler J, Black I, Ayer-LeLievre C (1985) Substance P-containing sensory nerves in the rat iris. Normal distribution, ontogeny and innervation of intraocular iris grafts. Neuroscience 15:519–528

Skofitsch G, Jacobowitz DM (1985a) Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. Peptides 6:509–546

Skofitsch G, Jacobowitz DM (1985b) Galanin-like immunoreactivity in capsaicin sensitive sensory neurons and ganglia. Brain Res Bull 15:191–195

Stefanini N, DeMartino C, Zamboni C (1967) Fixation of ejaculated spermatazoa for electron microscopy. Nature (Lond) 216:173–174

Tatemoto K, Rökaeus Å, Jörnvall H, McDonald TJ, Mutt V (1983) Galanin – A novel biologically active peptide from porcine intestine. FEBS Lett 164:124–128

Terenghi G, Polak JM, Probert L, McGregor GP, Fein GL, Blank MA, Butler JM, Unver WG, Zhang W-Q, Colde DF, Bloom SR (1982) Mapping, quantitative distribution and origin of substance P- and VIP-containing nerves in the uvea of guinea pig eye. Histochemistry 74:399–417

Terenghi G, Polak JM, Ghatel MA, Mulderry PK, Butler JM, Unger WG, Bloom SR (1985) Distribution and origin of calcitonin gene-related peptide (CGRP) immunoreactivity in the sensory innervation of the mammalian eye. J Comp Neurol 233:506–516

Tervo K, Tervo T, Eränkö O, Cuello AC (1981) Immunoreactivity for substance P in the Gasserian ganglion, ophthalmic nerve and anterior segment of the rabbit eye. Histochem J 13:435–443

Tervo T, Tervo K, Eränkö L (1982) Ocular neuropeptides. Med Biol 60:53–60

Tramu G, Pillez A, Leonardelli J (1978) An efficient method of antibody elution for the successive or simultaneous location of two antigens by immunocytochemistry. J Histochem Cytochem 26:322–324

Tschopp FA, Tobler PH, Fischer JA (1984) Calcitonin gene-related peptide in the human thyroid, pituitary and brain. Mol Cell Endocrinol 36:53–57

Uddman R, Alumets J, Ehinger B, Håkanson R, Loren I, Sundler F (1980) Vasoactive intestinal peptide nerves in ocular and orbital structures of the cat. Invest Ophthalmol Vis Sci 19:878–885

Yanagisawa M, Yagi N, Otsuka M, Yanaihara C, Yanaihara N (1986) Inhibitory effects of galanin on the isolated spinal cord of the newborn rat. Neurosci Lett 70:278–282

Accepted March 10, 1987