Cell Tissue Res (1981) 216:581–589

Cell and Tissue Research


# Ultrastructural Identification of Gastrin-like Immunoreactive Nerve Fibres in the Brain of *Xenopus laevis* by Means of Colloidal Gold or Ferritin Immunocytochemical Methods*

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**Summary.** By use of an anti-gastrin serum and colloidal gold- or ferritin-labelled sheep anti-rabbit $\gamma$-globulins, nerve fibres and nerve terminals containing a gastrin-like substance were characterized at the ultrastructural level in the median eminence of *Xenopus laevis*. These immunoreactive fibres contain neurosecretory granules displaying medium to high electron density and a mean diameter of 75 nm. Labelling intensity varies from granule to granule. This is the first demonstration at the ultrastructural level of the precise location of a gastrin-like hormone in the median eminence of a vertebrate.

**Key words:** Gastrin – CCK – Median eminence – Electron microscopy – *Xenopus laevis* (Amphibia, Anura)

Using light microscopic immunohistochemical methods (immunofluorescence and immunoperoxidase) several investigators have reported the presence of perikarya and nerve fibres reacting with anti-gastrin or anti-CCK sera in the brain and the hypophysis of various mammals (Straus et al. 1977; Larsson and Rehfeld 1979; Rehfeld et al. 1979; Innis et al. 1979; Lorén et al. 1979; Straus and Yalow 1979; Vanderhaegen et al. 1980), amphibians (Doerr-Schott et al. 1979), and fish (Notenboom et al. 1980).

In mammals only scattered perikarya and fibres of this type have been observed. In contrast, in fish and amphibians, the entire course of the fibres has been traced. In fish, two groups of perikarya occur in the postchiasmatic hypothalamus (more precisely in the *nucleus lateralis tuberis*); immunoreactive fibres emerge from the nucleus and end in the proximal *pars distalis* of the hypophysis. In amphibians, two groups of perikarya are located in the

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* Supported by the D.G.R.S.T., Contrat no 80.7.0242

*Acknowledgements:* The authors thank Mmes C. Lichte and R.O. Clauss for their skilful technical assistance and Mr. C. Chevalier for preparing the photographs

0302-766X/81/0216/0581/$01.80

postchiasmatic hypothalamus, i.e., in the *nucleus infundibularis ventralis* (Peute and Meij 1973); two dense fibre tracts emerge from this nucleus and terminate in the external layer of the median eminence. In amphibians, a second group of nerve endings has been observed in the prechiasmatic region, near the preoptic recess. Neither the site of origin of the corresponding perikarya, nor the precise course of their fibres have been defined.

The ultrastructure of the gastrin immunoreactive neurons has not been described. The present study extends previous light-microscopic investigations to the ultrastructural identification of the gastrin-immunoreactive fibres and endings in the median eminence. It was carried out with an indirect immunocytochemical method, using rabbit anti-gastrin serum as a first layer, and colloidal-gold or ferritin-labelled sheep anti-rabbit γ-globulins as the second layer.

## Materials and Methods

Six adult *Xenopus laevis*, of both sexes, were anaesthetized with methyltricaine sulfonate ($MS_{222}$; Sandoz, Paris, France) dissolved in water (0.1%), and then sacrified by decapitation. The median eminence of these animals was fixed in 2.5% glutaraldehyde in 0.1 M (pH 7.3) phosphate buffer for 2 h and postfixed in 1% osmium tetroxide 0.1 M (pH 7.3) veronal buffer for 1 h. The tissues were then dehydrated in graded ethanols and propylene oxide, and finally embedded in Araldite.

Serial semithin sections and ultrathin silver sections were cut. Semithin sections were mounted on glass slides. Ultrathin sections were mounted on simple slot grids covered with a carbon coated Formvar membrane. Subsequently, semithin sections and ultrathin sections were processed for immunohistochemistry.

*Immunological Reagents.* The rabbit anti-gastrin serum (Ga 18-3) was prepared and characterized according to Doerr-Schott et al. (1979). The fluorescent sheep anti-rabbit γ-globulins and the ferritin-labelled goat anti-rabbit γ-globulins were purchased from the Institut Pasteur (Paris, France) or Miles Co (Paris, France). Sheep anti-rabbit γ-globulins (kindly provided by Mr. A. Le Corre, Institut Pasteur, Paris) were labelled with colloidal gold according to Garaud et al. (1980a).

*Immunohistochemical Procedures.* For fluorescence microscopy, semithin sections were treated: 1) with a saturated solution of sodium hydroxide in ethanol (Lane and Europa 1965) at room temperature for 30 min, 2) with $H_2O_2$ (3 volume) for 30 min, and 3) stained using the indirect immunofluorescence method (Coons 1958).

For electron microscopy ultrathin sections were processed in microtest boxes used as staining dishes (Garaud et al. 1980b). Grids were floated, with sections facing down, in 20 µl of the following solutions: 1) clean distilled water, 2) rabbit anti-gastrin serum diluted at 1/50000 in phosphate buffer (0.01 M; pH 7.3) containing 1 mg/ml bovine serum albumine, for 1 h at room temperature, 3) phosphate buffer (3 washes of 5 min each), 4) colloidal gold- or ferritin-labelled γ-globulins for 1 h, 5) phosphate buffer (twice 5 min each), 6) distilled water (twice 5 min each). The first and last baths of clean distilled water were used to protect sections from dirt (Mollenhauer 1974). As etching with $H_2O_2$ did not modify the labelling intensity, in most experiments this step was omitted. Sections labelled with colloidal gold were counterstained with 2% aqueous uranyl acetate and lead citrate (Reynolds 1963). Ferritin-labelled sections were not counterstained. Sections were analyzed with a Philips 300 electron microscope.

*Immunocytochemical Controls.* Two types of controls were performed: 1) Controls of the non-specific adsorption of IgGs on tissues: replacement of the first antiserum with normal rabbit antiserum or phosphate buffer. 2) Controls due to the presence of identical amino-acid sequence in gastrin, cholecystokinin (CCK) and Peptavlon (synthetic C-terminal amino-acid sequence common to gastrin and CCK). To 1 ml of diluted anti-gastrin serum were added: a) 1 µg (0.48 µmol) 1–17 gastrin (ICI, Macclesfield, England); b) 150 µg (7.65 µmol) cholecystokinin with 20% purity (GIH, Karolinska Institute, Stockholm, Sweden); c) 100 µg (130.2 µmol) pentagastrin (Peptavlon, ICI).

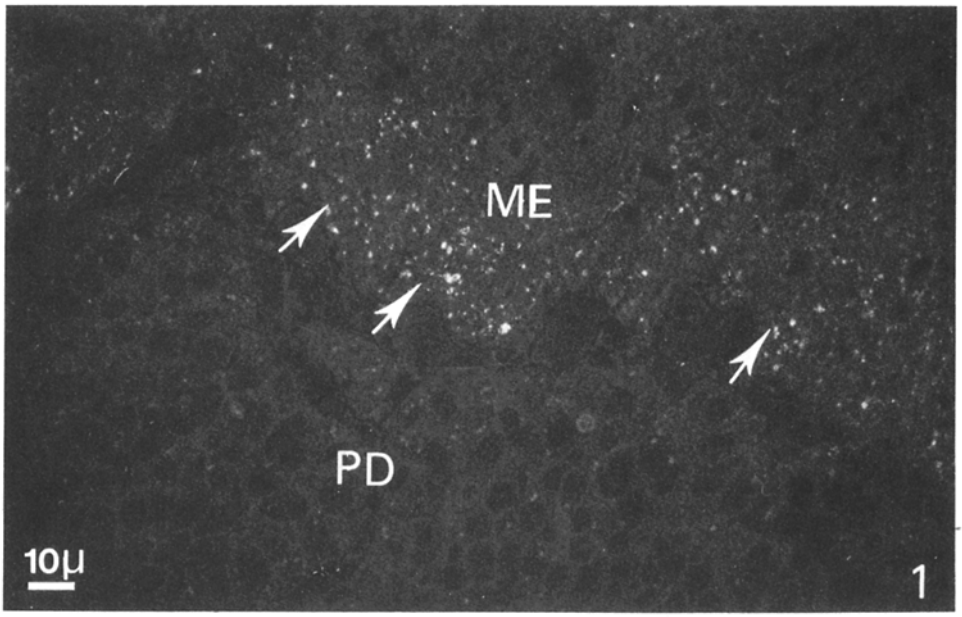
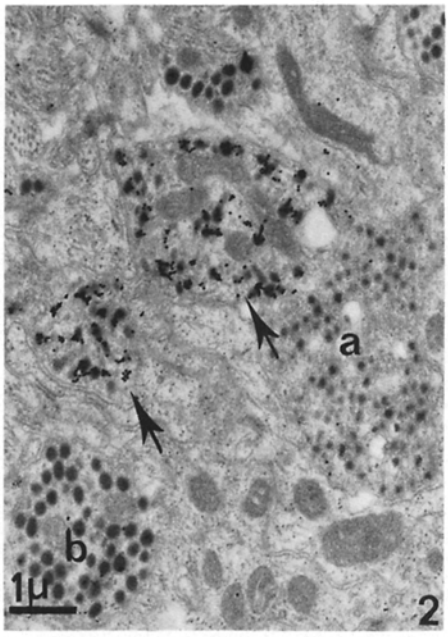



**Fig. 1.** Sagittal semithin section through the median eminence (*ME*) and the pars distalis (*PD*) of the hypophysis of *Xenopus laevis* showing numerous cross-sectioned immunofluorescent fibres (↗) in the external layer of the median eminence

## Results

Immunofluorescence seen in semithin sections was used to define the distribution of the immunoreactive gastrin-like substance in the median eminence. A strong positive reaction with the anti-gastrin serum was observed in the external layer of the median eminence cut in the sagittal plane. In this region, there are numerous immunofluorescent cross-sectioned fibres around the portal capillary loops. Higher numbers of scattered fibres were observed in the internal layer of the median eminence. No immunocytochemical reaction was seen in the neural lobe or in the adenohypophysis (Fig. 1).

Immuno-colloidal and immuno-ferritin methods were applied to ultrathin sections from the external layer of the median eminence where immunofluorescent fibres are abundant. Several isolated or aggregated particles of gold displaying high electron density occurred mainly on the dense neurosecretory granules of one particular type of nerve fibre or nerve ending (Figs. 2–4). These fibres or endings contained granules displaying medium to high electron density (Figs. 3, 4). The granules were enclosed by a membrane. A relatively clear space was generally observed between the dense core of the granule and the surrounding membrane. The diameter of the positive neurosecretory granules ranged from 50–100 nm. The density of gold particles on each granule varied from one immunoreactive fibre to another (Fig. 2). These fibres and nerve endings were scattered between non-immunoreactive elements which most likely contained other peptides or catechol-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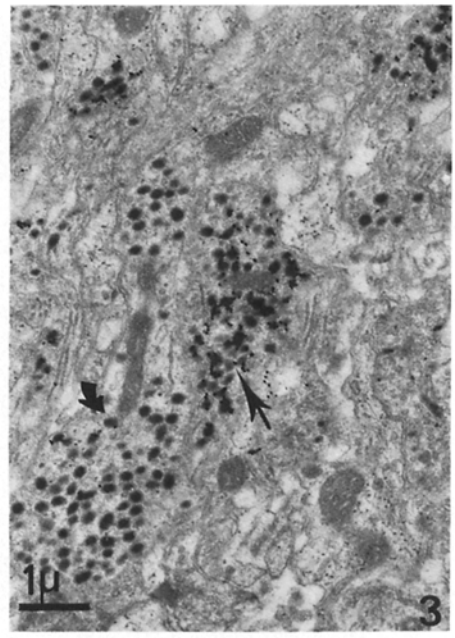
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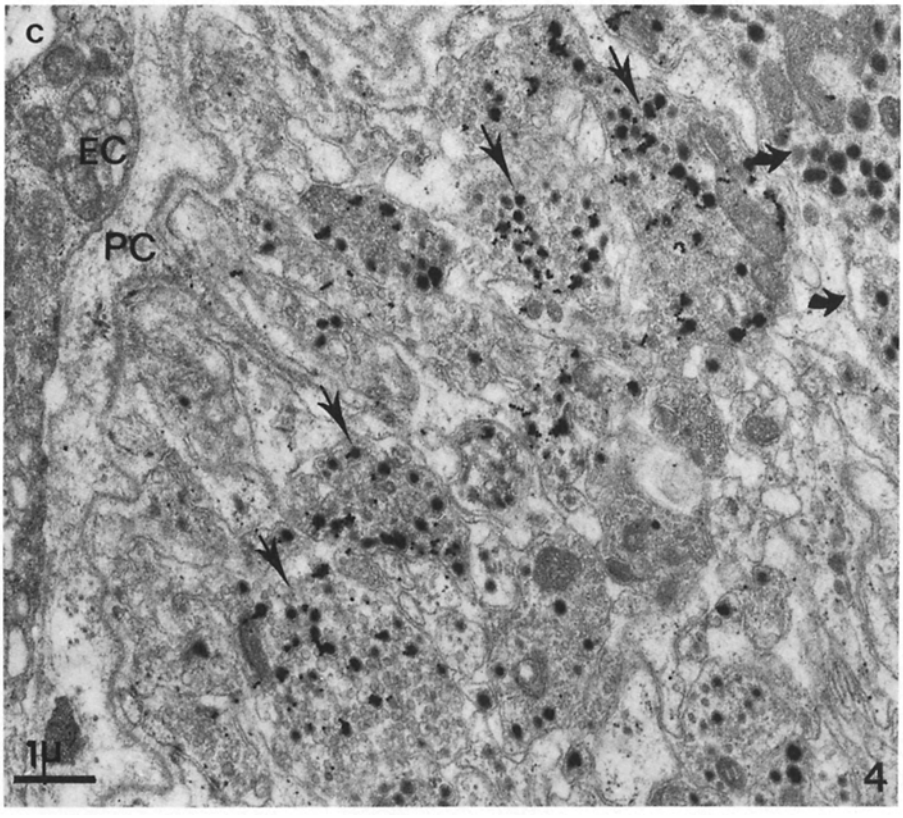
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amines (Figs. 2–4) (Doerr-Schott and Follénius 1970; Doerr-Schott and Dubois 1976).

As in other species (Rodríguez 1969; Doerr-Schott 1970) five different types of nerve fibres are present in the median eminence of *Xenopus laevis* (Doerr-Schott, in preparation). The immunoreactive axons contain granules with the ultrastructural characteristics (mean diameter, fairly high electron density) of fibre type 3. None of the other four fibre types observed in the external layer of the median eminence of this species was labelled (Figs. 2–4). However, some fibres that do not differ morphologically from the gastrin-immunoreactive elements do not exhibit a positive immunoreaction (Fig. 3). These fibres may either have lost their gastrin content or may contain a different neuropeptide.

A few immunoreactive nerve fibres were also observed in the inner layer of the median eminence, which contained mainly vasotocinergic, mesotocinergic (Van Vossel-Daeninck et al. 1979; Dierickx 1980) and catecholaminergic nerve elements (Doerr-Schott and Follénius 1970). Some of the immunoreactive profiles occured: 1) between the ependymal cells lining the floor of the third ventricle, or 2) near the ependymal processes crossing the median eminence. Ependymal cells did not show a positive reaction.

The results obtained with ferritin-labelled $\gamma$-globulins are comparable to the above-described findings, except that the tracer is even more clearly located over the neurosecretory granules (Fig. 7).

Specificity controls were based on the comparison of successive serial sections treated under the same conditions either with the untreated anti-gastrin serum or the serum to which 2–17 gastrin, CCK or Peptavlon were added. Inhibition of the reaction was observed with each of these peptides (Figs. 5, 6).

## Discussion

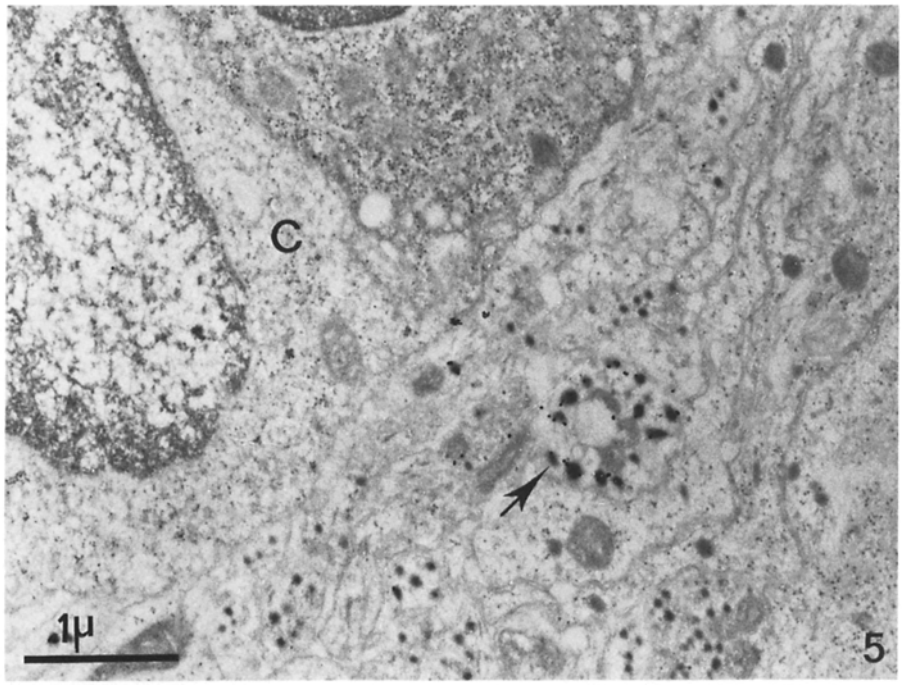
The well developed median eminence of anurans is responsible, via neurovascular channels, for the hypothalamic control of the anterior hypophysis. Neurovascular connections in the median eminence were studied by means of various cytological techniques, both light and electron microscopically. During the last decade, the use of antisera against various peptides has enabled the immunocytochemical localisation of different types of nerve fibres in the median eminence of amphibians,

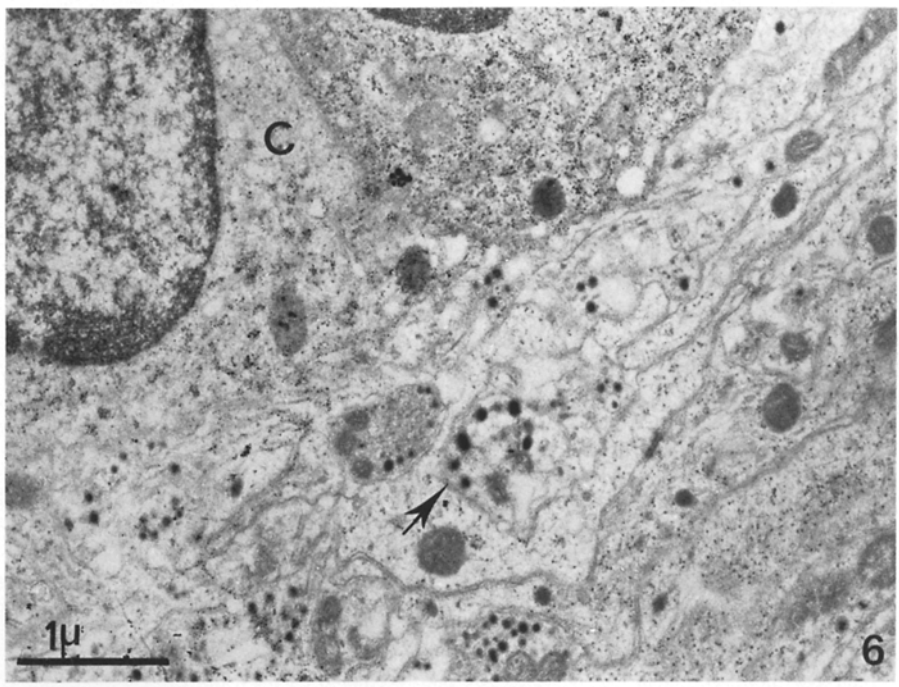
**Figs. 2–4.** Thin sections of glutaraldehyde/$OsO_4$-fixed median eminence stained immunocytochemically with anti-gastrin serum (Ga 18-3) and gold-labelled $\gamma$-globulins. Sections counterstained with uranyl acetate and lead citrate. Dilutions: Ga 18-3 : 1/50,000, gold-labelled $\gamma$-globulins : 1/20

**Fig. 2.** Two immunopositive nerve fibres (↗) revealed by gold particle deposits on the neurosecretory granules. Two other types of axons (*a* and *b*) contain unstained granules

**Fig. 3.** Gastrin-immunoreactive fibre (↗) near a non-reacting fibre (↗) and displaying a similar morphology

**Fig. 4.** A portion of the external layer of the median eminence. Many nerve endings (↗) containing both small immunoreactive secretory granules and synaptic vesicles are observed near a capillary (*c*); ↗ non-reacting fibres. *EC* endothelial cells; *PC* pericapillary space




**Figs. 5, 6.** Two adjacent sections of the median eminence of *Xenopus laevis* counterstained with uranyl acetate and lead citrate

**Fig. 5.** Section treated with anti-gastrin serum. Note an immunoreactive nerve fibre (↗) (type 3) near the cell (*c*)

**Fig. 6.** Adjacent control section treated with anti-gastrin serum inhibited with Peptavlon. No immunoreaction on type 3 fibre (↗)

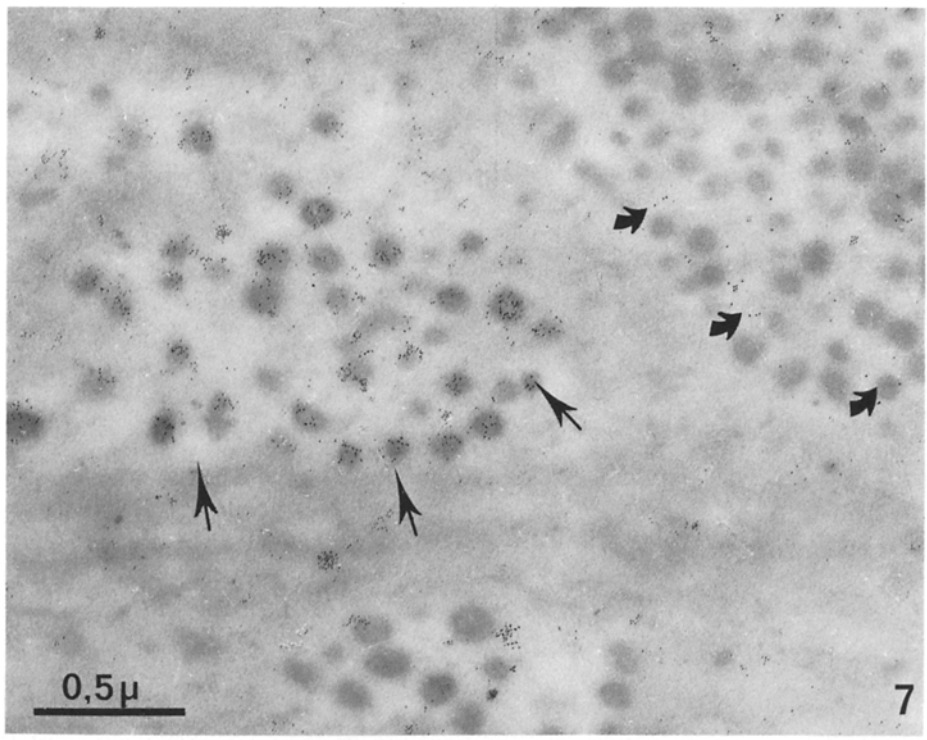



**Fig. 7.** Gastrin-like immunoreactive fibre (↗) revealed with anti-gastrin serum and ferritin-labelled γ-globulins; note the adjacent non-immunoreactive axon (↗). Section is not counterstained

particularly elements containing luteinizing hormone releasing hormone (LHRH) (Doerr-Schott and Dubois 1976), somatotrophin release inhibiting factor (SRIF) (Doerr-Schott unpublished results), vasotocin, and mesotocin (Dierickx and Vandesande 1977).

The ultrastructure of the median eminence of *Xenopus laevis* resembles closely that of bufonid (Rodríguez 1969) and ranid species (Doerr-Schott 1970). Based on the diameter of the neurosecretory granules, five categories of fibres can be distinguished and each type of granules is assumed to be associated with a particular agent. The recent application of immunocytochemical technique in the study of the median eminence of *Xenopus laevis* has enabled the ultrastructural identification of the GnRH (gonadotropin-releasing hormone)-containing axons. These elements display neurosecretory granules of various shapes, approximately ~90 nm in diameter (Doerr-Schott et al. 1978). They differ morphologically from the gastrin-immunoreactive fibres described in the present communication; the latter contain smaller granules (mean diameter 75 nm). The gastrin-immunoreactive fibres are adjacent to non-immunoreactive elements, which may contain granules similar in their aspect and morphology to the granular inclusions of the immunoreactive fibres. This result indicates that the criteria of identification (diameter, shape, density of neurosecretory granules) usually applied for definition

and classification of the different axonal types in the median eminence are only of limited value. Under these circumstances, immunocytological techniques used in combination with electron microscopy are of particular interest, since they enable direct identification of the intraaxonal neuropeptides without involving measurements of granules diameters.

As outlined in a previous paper (Doerr-Schott et al. 1979), the antigenic sites reacting with the anti-gastrin serum used are common to gastrin and CCK; thus the immunoreactive material revealed with the gastrin antiserum might also be a CCK-like substance.

The physiological role of the immunoreactive fibres characterized in the present study is still enigmatic. Concerning mammals, however, various hypotheses have been put forward in connection with this system. In vivo and in vitro experiments suggest an intervention of cholecystokinin on gonadotropin, prolactin, thyreotropin (Vijayan et al. 1979) and on growth hormone release (Vijayan et al. 1979; Morley et al. 1979). Other in vivo experiments showed that injection of cholecystokinin suppresses feeding (Della-Fera and Baile 1979; Falasco et al. 1979). Finally, Bitar et al. (1979) underlined the "interaction of acetylcholine and cholecystokinin with dispersed smooth muscle cells."

In amphibians, experimental studies are needed to elucidate the physiological role of the gastrin-like material revealed in the brain. Previous experiments suggested that hypophysiotropic centres, in particular a gonadotropic centre (Dierickx 1966) and a feeding centre (Dierickx 1969), are located in the *pars ventralis* of the *tuber cinereum*. In view of these experimental results it remains to be established whether the centre containing gastrin-like material is identical or not to one of these previously defined centres.

## References

Bitar KN, Zfass AM, Makhlouf GM (1979) Interaction of acetylcholine and cholecystokinin with dispersed smooth muscle cells. Am J Physiol (Endocrinol) 6:E172–E177

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

Della-Fera MA, Baile CA (1979) Cholecystokinin octapeptide: Continuous picomole injections into the cerebral ventricules of sheep suppress feeding. Science 206:471–473

Dierickx K (1966) Experimental identification of a hypothalamic gonadotropic centre. Z Zellforsch 74:53–79

Dierickx K (1969) Hypothalamo-hypophysial regulation of food intake in *Rana temporaria*. Gen Comp Endocrinol 13:361–366

Dierickx K (1980) Immunocytochemical localization of the vertebrate cyclic nonapeptide neurohypophyseal hormones and neurophysins. Int Rev Cytol 62:119–185

Dierickx K, Vandesande S (1977) Immunocytochemical demonstration, in the external region of the amphibian median eminence, of separate vasotocinergic and mesotocinergic nerve fibres. Cell Tissue Res 177:47–56

Doerr-Schott J (1970) Etude au microscope électronique de la neurohypophyse de *Rana esculenta* L. Z Zellforsch 111:413–426

Doerr-Schott J, Follénius E (1970) Identification et localisation des fibres aminergiques dans l'éminence médiane de la grenouille verte (*Rana esculenta* L.) par autoradiographie au microscope électronique. Z Zellforsch 111:427–435

Doerr-Schott J, Dubois MP (1976) LHRH-like system in the brain of *Xenopus laevis* Daud. Immunohistochemical identification. Cell Tissue Res 172:477–486

Doerr-Schott J, Clauss RO, Dubois MP (1978) Localisation immunohistochimique au microscope électronique d'une hormone GnRH dans l'éminence médiane de *Xenopus laevis* Daud. C R Acad Sci (Paris) 286:477–479

Doerr-Schott J, Garaud JC, Clauss RO (1979) Immunohistochemical localization of a gastrin-like peptide in the brain of an amphibian, *Xenopus laevis* Daud. Cell Tissue Res 203:65–78

Falasco JD, Smith GP, Gibbs J (1979) Cholecystokinin supresses sham-feeding in the rhesus monkey. Physiol Behaviour 23:887–891

Garaud JC, Doeffel M, Stock C, Grenier JF (1980a) G-cells and other gastrin-like immunoreactive cells of the human antrum. Cell Tissue Res (submitted for publication)

Garaud JC, Stock C, Grenier JF (1980b) Reproducibility in staining ultrathin sections. Some improvements. Stain Technol (submitted for publication)

Innis RB, Corrès FMA, Uhl GR, Schneider B, Snyder BH (1979) Cholecystokinin octapeptide-like immunoreactivity: Histochemical localization in rat brain. Proc Natl Acad Sci USA 76:521–525

Lane BP, Europa DL (1965) Differential staining of ultrathin sections of epon embedded tissues for light microscopy. J Histochem Cytochem 13:579–582

Larsson LI, Rehfeld JF (1979) Localization and molecular heterogeneity of cholecystokinin in the central and peripheral nervous system. Brain Res 165:201–218

Lorén I, Alumets J, Häkanson R, Sundler F (1979) Distribution of gastrin and CCK-like peptides in rat brain. Histochemistry 59:249–257

Mollenhauer HH (1974) Poststaining sections for electron microscopy. Stain Technol 48:305–308

Morley JE, Melmed S, Briggs J, Carlson HE, Hersman JM, Solomon TE, Lamers C, Damassa DA (1979) Cholecystokinin octapeptide releases growth hormone from the pituitary in vitro. Life Sci 25:1201–1306

Notenboom CD, Garaud JC, Doerr-Schott J, Terlou M (1981) The localization by immunofluorescence of a gastrin-like substance in the brain of the rainbow trout, *Salmo gairdneri*. Cell Tissue Res 214:247–255

Peute J, Meij JCA (1973) Ultrastructural and functional aspects of the nucleus infundibularis ventralis in the green frog, *Rana esculenta*. Z Zellforsch 144:191–217

Rehfeld JF, Goltermann N, Larsson LI, Emson PM, Lée CM (1979) Gastrin and cholecystokinin in central and peripheral neurons. Fed Proc 28:2325–2329

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

Rodríguez EM (1969) Ultrastructure of the neurohaemal region of the toad median eminence. Z Zellforsch 93:182–212

Straus E, Yalow RS (1979) Gastrointestinal peptides in the brain. Fed Proc 38:2320–2324

Straus E, Muller JE, Choi HS, Paronetto F, Yalow R (1977) Immunohistochemical localization in rabbit brain of a peptide resembling to COOH-terminal octapeptide of cholecystokinin. Proc Natl Sci USA 74:3033–3034

Vanderhaegen JJ, Lotstra F, De Mey J, Gilles G (1980) Immunohistochemical localization of cholecystokinin- and gastrin-like peptides in the brain and hypophysis of the rat. Proc Natl Acad Sci USA 77:1190–1194

Vijayan E, Samson WK, McCann SM (1979) In vivo and in vitro effects of cholecystokinin on gonadotropin, prolactin, growth hormone and thyrotropin release in the rat. Brain Res 172:295–302

Vossel-Daeninck J van, Dierickx K, Vossel A van, Vandesande F (1979) Electron microscopic immunocytochemical demonstration of separate vasotocinergic and mesotocinergic nerve fibres in the median eminence of the frog hypophysis. Cell Tissue Res 204:29–36

Accepted December 29, 1980