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Chromogranin Immunoreactivity in the Central Nervous System. Immunochemical Characterisation, Distribution and Relationship to Catecholamine and Enkephalin Pathways

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1. INTRODUCTION

The catecholamine storing secretory granules of the chromaffin cells in the adrenal medulla contain water-soluble proteins which have been collectively called chromogranins[5]. Few of the proteins have established roles: one, dopamine β-hydroxylase (DBH), is involved in the biosynthesis of catecholamines and others include various forms of the enkephalin precursor (see ref. 106). The proteins which have been assigned specific roles account for a small fraction of the total soluble proteins. Roughly 40–50% of the mass of water-soluble proteins is accounted for by one protein, called chromogranin A[80,87]. There is a wealth of data relating to the biochemical properties of this protein, the most pertinent to this study being its relative molecular mass (M_r) of 70,000–80,000 and its richness in acidic amino acids (see ref. 116). It is also present in noradrenergic sympathetic nerve vesicles (see ref. 51), and it is secreted from chromaffin cells and sympathetic neurons when the amines are released[5,88,89]. The functional role of chromogranin A in the adrenal medulla and sympathetic nerves is unknown. Attempts to establish the role of the protein have focused on its association with catecholamines and it was proposed to be an important component in maintaining the high concentration of amines in vesicles (see ref. 88). However, it has not been possible to show unambiguously that its presence is a major requirement of amine storage (see refs. 51, 116). Recent work has shown that chromogranin immunoreactive material is not exclusively associated with catecholamines but is also present in several endocrine tissues where it is localized in polypeptide-hormone producing cells[15,16,66,67]. Chromogranin immunoreactive material has also been found in serum[64], suggesting that it may be secreted from the cells.

The presence of chromogranin in endocrine cells which secrete peptide hormones indicates that its role is not confined to aminergic tissues, and its secretion suggests that the protein, or possibly peptides

derived from it, may act as hormone-like messengers. Many of the polypeptides synthesized by endocrine cells are also widely distributed in the nervous system[42,49], where they are believed to act as neurotransmitters or modulators. It follows that if the chromogranins were also present in the central nervous system they may serve to influence neuronal activity.

We have tested the hypothesis that a wider role for some of the chromogranins should be linked to a wide but selective distribution in the nervous system. As a first step we have studied the distribution of chromogranin-like immunoreactivity using a newly characterized antiserum raised against purified chromogranin A. The antiserum also reacts with closely related polypeptides. In order to relate the distribution of chromogranin to established neuroactive substances, we have also studied its distribution in relation to immunoreactive Leu-enkephalin, Met-enkephalin, Met-enkephalin-Arg^6-Phe^7 tyrosine hydroxylase (TH), and DBH. A preliminary report of some of these findings has been published[97].

2. MATERIALS AND METHODS

2.1. *Isolation of chromogranin A*

The soluble proteins of bovine adrenal chromaffin granules were prepared according to the method of Fischer-Colbrie et al.[27]. The chromogranin A was then separated from the rest of the proteins using disc gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)[25]. After staining the proteins with Coomassie Blue, the area containing chromogranin A was removed and the protein electro-eluted from the pieces of gel[27]. This material was used to produce antiserum 1.

A second procedure was also used: the soluble proteins were isolated as before except that phenylmethylsulphonyl fluoride (50 μM) was included in all solutions to inhibit serine-type protein hydrolases. Dopamine β-hydroxylase (DBH) and glycoprotein III were then removed by passing the chromogranins through columns of concanavalin–A-sepharose (Pharmacia) and wheat-germ–agglutinin–sepharose (Pharmacia). The final effluent, containing the chromogranin A, was then applied to an HPLC gel permeation column, BioSil TSK-400 (Bio Rad), equilibrated with sodium acetate buffer, 20 mM, pH 6.5, containing 40 mM sodium chloride and eluted with the same buffer at a flow rate of 0.3 ml/min. Fractions enriched in chromogranin A were subjected to SDS slab gel electrophoresis[50]. After staining, gel pieces containing chromogranin A were cut out and the protein allowed to diffuse from the gel. The SDS was removed by precipitation with acetone[37]. This material was used to produce antiserum 2.

2.2. *Production of antiserum to chromogranin*

Chinchilla rabbits were injected intradermally with 800 μl of a 1:1 emulsion of chromogranin A (100 μg) and Freund's complete adjuvant. Initial injections were given on day 1 and 14 and boosters, using Freund's incomplete adjuvant, were given at monthly intervals. Rabbits were bled 10 days after each booster injection.

2.3. *Characterization of the antiserum to chromogranin*

2.3.1. *One dimensional immunoblots*

Immunoblotting was carried out on sheets of nitrocellulose paper (Sartorius, Gottingen, F.R.G.) by first separating the soluble proteins in tissue extracts using SDS polyacrylamide gel electrophoresis[50]. The proteins were then transferred to sheets of nitrocellulose by electrophoresis[104] overnight at 4 °C with a voltage gradient measured to be 15 V/cm. The sheets were then incubated sequentially in the following solutions: 30 min at room temperature in 3% gelatine blocking solution; overnight at 4 °C in chromogranin antiserum diluted 1:800 (antiserum 1) or 1:200 (antiserum 2) with 20% normal goat serum in 10 mM phosphate buffered isotonic saline containing 10 mM Tris, pH 7.4 (TPBS); 3 × 10 min washes at room temperature with TPBS; 30 min at room temperature in biotinylated affinity-purified, goat-anti-rabbit immunoglobulin (Vector Labs., CA, U.S.A.), diluted 1:200 in 1% normal goat serum in TPBS; 3 × 10-min washes in TPBS; 30 min in avidin-peroxidase complex (Vector); 3 × 10-min washes; 5 min in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Serva, Heidelberg), in Tris buffer (50 mM, pH 7.4). Hydrogen peroxide was then added (0.01% final concentration).

2.3.2. *Two dimensional immunoblots*

Antisera were also tested first by isoelectric focusing extracts of protein in one direction followed by SDS gel electrophoresis in the other[70]. Immunoblotting was performed using a modified[15] version of the method of Burnette[11]. After transfer of the proteins, the nitrocellulose sheets were treated with dilute bovine serum albumin and goat serum (2% v/v) for one h, then for 3 h with CHR-antiserum (1:250) diluted in normal goat serum (2% v/v). After 5 × 10-min washes, the sheets were incubated in ^{125}I labeled protein A (0.2 μCi/ml) diluted in goat serum (2% v/v). After 5 × 10-min washes the nitrocellulose was processed for autoradiography[11].

2.4. *Preparation of tissue extracts*

Sheep brains were obtained from a local slaughterhouse within 20–30 min of death and transported on ice to the laboratory. Samples of tissue were then removed from the following areas of brain: olfactory tubercle, visual cortex (area 17), frontal cortex, lateral geniculate nucleus, superior colliculus, putamen, globus pallidus, substantia nigra, the periventricular area of the thalamus, the ventrolateral nucleus of the thalamus, the hypothalamus, amygdala, the corpus callosum, subcortical white matter, the dentate gyrus plus the CA3 region of the hippocampus, the CA1 region of the hippocampus, cerebellar cortex, the deep cerebellar nuclei and the red nucleus. Samples were also taken from the anterior pituitary and the neural lobe, the infundibulum, the neural stalk and the dorsal and ventral horns of the spinal cord.

Samples from the hippocampus (whole), the dorsal horn and ventral horn of the spinal cord and the adrenal medulla were taken from cattle.

The tissues were homogenized in 5 vols. of Tris buffer (5 mM, pH 7.4) using a Polytron homogenizer (max speed for 15 s). The samples were frozen and thawed 3 times and then centrifuged in an air-driven ultracentrifuge ('Airfuge' Beckman, 30 psi for 5 min; 165,000 rpm). These high-speed supernatants were prepared for electrophoresis[50].

Chromaffin granules were isolated from homogenates of the adrenal medulla by the method of Smith and Winkler[86]. The chromogranins were extracted by suspending the granule pellet in a hypotonic Tris buffer (5 mM; pH 7.4) and freezing the suspension. After thawing and centrifugation, the chromogranins were dialyzed against a large excess of the same buffer, with frequent changes, for 24 h at 4 °C.

Protein was measured according to the method of Bradford[8] using bovine γ-globulin (Sigma) as the standard. All reagents used throughout these studies were of analytical grade.

2.5. *Other antisera*

Antiserum to tyrosine hydroxylase was a gift from Drs. J. F. Powell and A. D. Smith, and its characterization has been described[31]. The preparation of the antibodies to dopamine β-hydroxylase has been described elsewhere[28].

Antisera to Met-enkephalin (Code no. L146) and Met-enkephalin-Arg^6-Phe^7 (Code no. L150) were gifts from Dr. G. J. Dockray. It has been shown[114] that the immunoreactivity of serum L146 in histological sections is not affected by prior adsorption of the serum to Met-enkephalin-Arg^6-Phe^7, and the immunoreactivity of serum L150 is not affected by prior adsorption of the serum to Leu- or Met-enkephalin.

Antiserum to Leu-enkephalin was a gift from Dr. M. Tohyama. This antiserum cross-reacts less than 1% with Met-enkephalin, less than 0.01% with dynorphin and ACTH and there was no cross-reaction with a large number of other peptides in radio-immunoassay[82].

2.6. *Animals and preparation of tissue sections*

Specimens for immunohistochemistry were obtained from two oxen and two cows (*Bos taurus*, red-polled short-horn breed); from 5 sheep (*Ovis aries*, merino/suffolk cross breed); 3 rats (albino, Porton strain), two guinea pigs (IMVS-coloured strain) and one rabbit (New Zealand white strain). Brains and adrenal glands from the cattle and 4 of the sheep were collected at the abattoir. Thick slices (3–8 mm) were cut from the brain and adrenals and were immersed in fixative within 20 min of the animal's death. One sheep was perfused. It was anesthetized with sodium thiopentone (20 mg/kg i.v.) intubated and artificially ventilated with a mixture of halothane (1.5%), oxygen (50%) and air. The descending aorta was clamped and the animal was perfused through

the heart, first with saline then by approximately 15 liter of fixative.

All other animals were anesthetized with chloral hydrate (35 mg/kg) and perfused through the heart with saline followed by fixative. After initial trial experiments with different fixatives, the combined paraformaldehyde-glutaraldehyde-picric acid fixative[92], was found to give satisfactory results for both light and electron microscopy. It was used for all the experiments.

After perfusion, tissue slices were immersed in the same fixative for 1–2 h. Specimens fixed only by immersion were left in the fixative for 6–8 h with continuous agitation. The fixed tissues were processed in 3 different ways: (1) for post-embedding, light microscopic immunohistochemistry, small blocks of tissue (up to 5 × 5 × 5 mm) were washed free of fixative in 0.1 M sodium phosphate buffer (pH 7.4; PB), dehydrated and embedded in Durcupan ACM (Fluka) resin. Sections either 0.5- or 1-μm thick were cut from these blocks and mounted on egg-albumin coated slides. Post-embedding immunohistochemistry was carried out as described previously[96] using the same reagents as for pre-embedding immunohistochemistry. (2) For pre-embedding light microscopic immunohistochemistry, 50–80-μm thick sections (up to 10 cm^2) were cut using a Vibratome (Oxford Instruments) and washed free of fixative. The perfused sheep brain was cut into slices in the coronal plane and sections were cut serially to represent most brain areas. The sections were then processed for immunohistochemistry (see below). (3) For combined light and electron microscope immunocytochemistry, small blocks of tissue (max 5 × 5 × 8 mm) were immersed in sucrose, frozen and thawed as described earlier[92], cut on a Vibratome (70 μm) and washed free of fixative in PB.

2.7. *Pre-embedding immunohistochemistry*

All solutions used in the preparation of sections for light microscopy only, contained 0.5% Triton X-100, but it was not used for sections prepared for electron microscopy. TPBS was used for diluting antisera and for washes. The unlabeled antibody peroxidase–antiperoxidase method[99] was used to locate immunoreactivity.

Incubations were performed in the following sequence: 10 min in TPBS; 1 h in 20% normal sheep serum; one day at 4 °C in primary antiserum; 3 × 40 min washes; 3–8 h in 1:50 sheep anti-rabbit IgG antiserum (Silenus Lab., Australia); 3 × 40 min washes; overnight in 1:100 rabbit peroxidase–antiperoxidase (Bioproducts, Belgium, or Miles Labs.); 3 × 40-min washes. Following a further wash in PB, the sections were incubated for 30 min in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 50 mM Tris buffer (pH 7.4) followed by a 10-min incubation in the same solution containing 0.01% H_2O_2. After further washes (2 × 20 min) in PB, the sections to be used for light microscopy only were mounted on gelatine-coated slides, dipped in 0.01% OsO_4 solution, dehydrated and covered in XAM neutral mounting media.

Sections for combined light and electron microscopy were also washed in PB, kept for 1 h in 1% OsO_4, washed in PB, dehydrated and mounted on slides in Durcupan ACM resin.

Correlated light and electron microscopy was carried out as described earlier[92]. The electron microscopic sections were not stained with heavy metals, but 1% uranyl acetate was included into the 70% ethanol during dehydration.

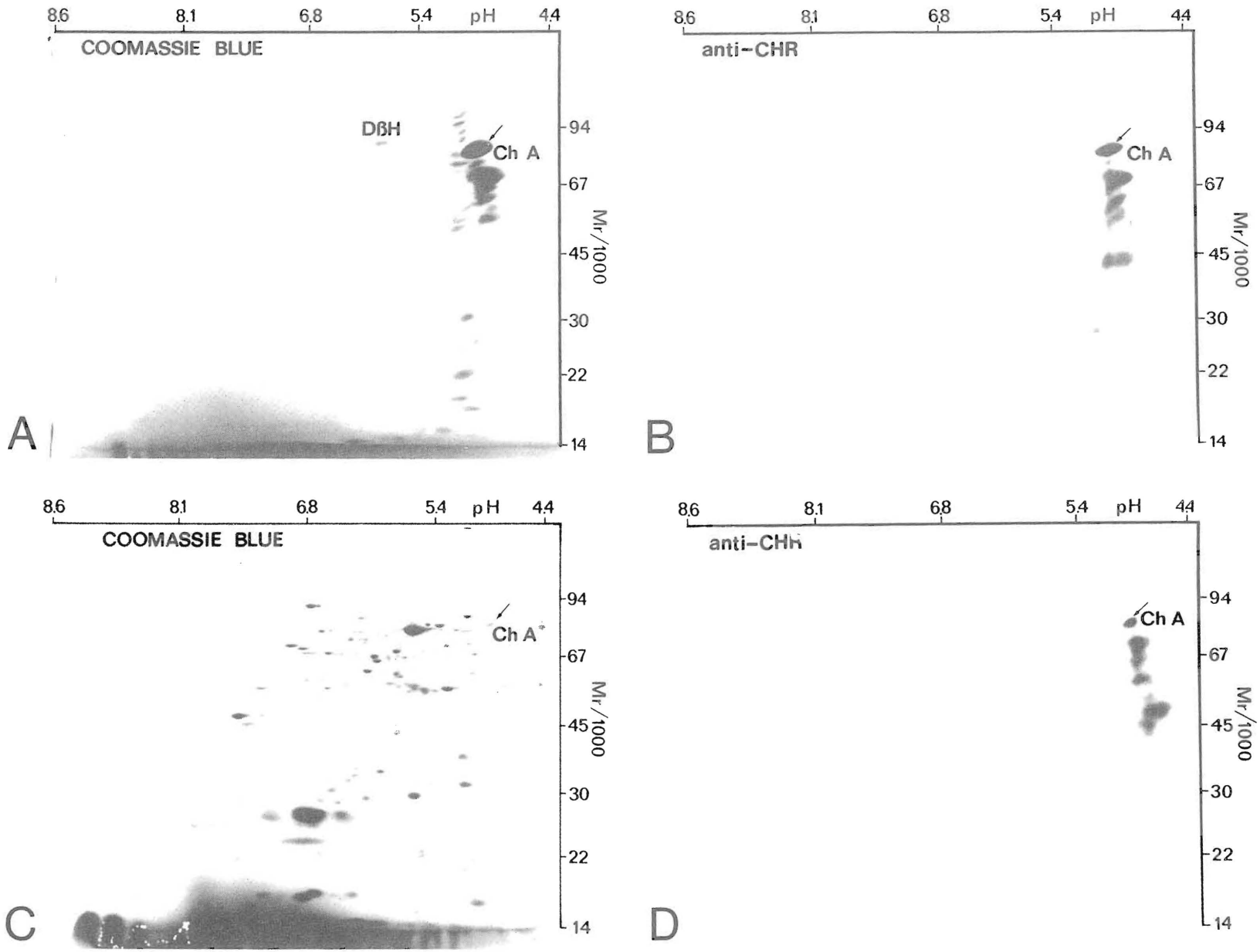
2.8. *AChE enzyme histochemistry*

Acetylcholinesterase activity was demonstrated using acetylthiocholine as the substrate[48]. A modified version of the original procedure was used[52]. BW284C51 (10^{-4} M) abolished the enzyme reaction confirming that the reaction was due to the presence of AChE[84].

2.9. *Controls for specificity of the immunohistochemical reaction*

Method specificity was tested by omitting the DAB or H_2O_2 from the incubation solution. Sections were also incubated in non-immune rabbit serum at the same dilutions as the primary antisera. Under these conditions no peroxidase reaction endproduct was found in neurons or neuronal processes.

Antisera to Leu-enkephalin, Met-enkephalin and Met-enkephalin-Arg^6-Phe^7 were absorbed with the respective peptides (10^{-5} M) overnight at 4 °C. No staining could be detected in sections treated with the


8.6
8.1
6.8
5.4
pH
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COOMASSIE BLUE
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67
45
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Mr/1000
A
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Ch A
B
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anti-CHR
Ch A
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absorbed antiserum.

We have studied the possibility that the immunostaining in the brain could have been caused by contaminating antibodies directed against impurities in the chromogranin A preparation used for immunisation. Extensive absorption tests were carried out against synthetic peptides as well as to tissue extracts to establish if the staining obtained in the adrenal and in the brain were affected in a parallel fashion. The tests were performed using sections of the adrenals from ox, sheep and rat and sections of the hippocampi of sheep and ox using post-embedding conditions. The hippocampus was chosen because it was one of the most strongly CHR-immunoreactive structures in the central nervous system.

The antiserum to chromogranin at final dilutions of 1:200, 1:400 and 1:800 was treated with several peptides and tissue extracts. The protein, peptide or tissue extract was mixed at the final concentration indicated and incubated overnight at 4 °C. Peptides included Met-enkephalin (Sigma, 10^{-5} M); Leu-enkephalin (Sigma, 10^{-5} M); Met-enkephalin-Arg^6-Phe^7 (Sigma, 10^{-5} M); dynorphin$_{1-17}$ (Peninsula Lab. 10^{-4}, 10^{-5}, 10^{-6} M); BAM22P (Peninsula Lab. 10^{-4} M, 10^{-6} M); bovine carbonic anhydrase (10^{-4} M, 10^{-5} M, Sigma Cat. no. C7500); a high speed supernatant from bovine hippocampus (at protein concentrations of 15.7, 13.5, 9, 4.5, 0.9, 0.18, 0.036, 0.0072 mg/ml) and the chromogranin-containing high speed supernatant from a bovine chromaffin granule lysate (at protein concentration of 100, 20, 4, 0.8 μg/ml). The sera were then centrifuged for 5 min at 165,000 rpm in an Airfuge and the supernatant used for immunohistochemical staining.

3. RESULTS

3.1. *Characteristics of the antiserum to chromogranin*

3.1.1. *Adrenal medulla*

Two-dimensional analysis of bovine chromogranins revealed that they comprised a group of acidic polypeptides with a restricted range of isoelectric points (4.8–5.2), but different relative molecular masses (M_r 14,000–100,000, Fig. 1A). The only exception was DBH which had a pI near 6 (Fig. 1A). The most abundant protein was identified as chromogranin A (Fig. 1A, apparent M_r approximately 80,000).

The two antisera, produced in response to immunisation with chromogranin A, were used for immunostaining of nitrocellulose replicas of gels. Both antisera reacted with a set of polypeptides including chromogranin A. The peptides had an almost identical pI (Fig. 1B). Another soluble chromaffin granule protein, DBH, did not react with either of the sera (Fig. 1B). Similarly, many of the minor proteins illustrated in Fig. 1A did not react with these antisera. However, at least 3 polypeptides with isoelectric points different from chromogranin A, did react with antiserum 1 but not 2. Since antiserum 1 was produced by immunizing with an immunogen that had undergone fewer purification steps, these are regarded as contaminants. One contaminating polypeptide that was immunoreactive with antiserum 1 but not 2 was identified as carbonic anhydrase.

One-dimensional analysis of the bovine chromaffin granule lysate showed a similar pattern of bands to the two-dimensional analysis. Heavily loaded gels showed a series of immunoreactive polypeptides ranging in size from M_r 80,000 to smaller peptides running in the buffer front. On less heavily loaded gels two major bands were stained and these corresponded in mobility to chromogranin A and to a slightly smaller protein (Fig. 2C, lanes 1 and 2). An immunoblot of the soluble extract made from an homogenate of the whole sheep adrenal medulla gave a pattern identical to bovine chromaffin granule lysate.

3.1.2. *Definition of chromogranin immunoreactivity*

Both our antisera reacted with a family of polypeptides related to chromogranin A, as well as with chromogranin A itself. The term chromogranin immuno-

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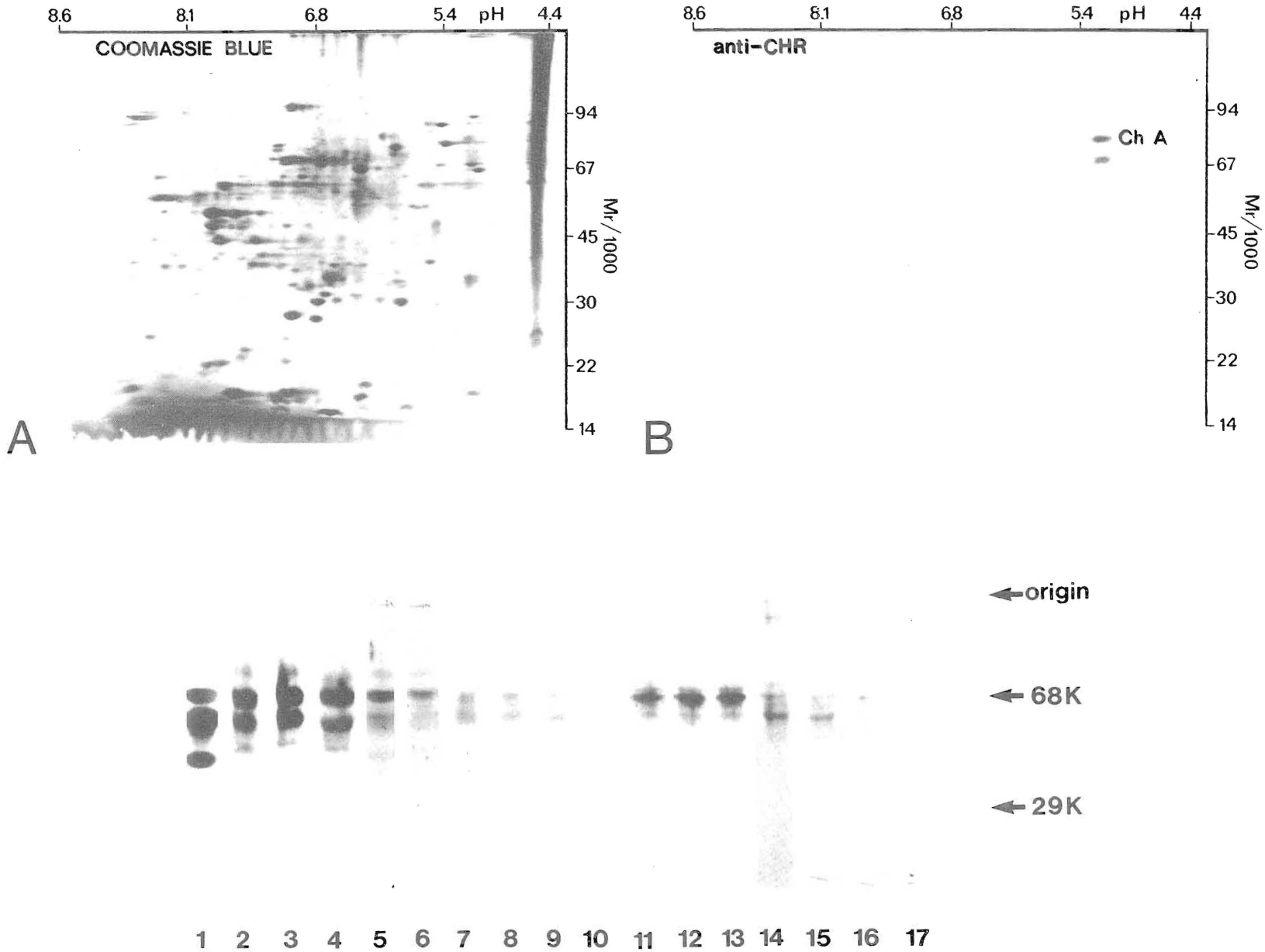
Fig. 1. Analysis of CHR-immunoreactivity in bovine chromaffin granule lysates (A, B) and in homogenates of bovine anterior pituitary (C, D). Isoelectric focusing was carried out in rod gels which developed a pH gradient between 8.6 and 4.4. The proteins were then electrophoresed into SDS gels. Marker proteins were used to calibrate the gels. The total proteins in the lysate and in pituitary were stained with Coomassie brilliant blue (A, C) and gels run in parallel were used to produce immunoreplicas. The latter were stained with anti-chromogranin serum (B, D). Proteins were immunostained which had different sizes, one was chromogranin A (ChA, arrowed). They all had similar isoelectric points in the range 4.8–5.2. Dopamine β-hydroxylase (DBH) did not react.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30
22
14
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A
anti-CHR
Ch A
B
origin
68K
29K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

reactivity will therefore be used to indicate that a family of related but differently sized polypeptides, all having a very similar isoelectric point, are recognized by the antisera used in these studies.

3.2. *Chromogranin immunoreactive proteins in brain and pituitary*

3.2.1. *Bovine pituitary and hippocampus*

High speed supernatant fractions from homogenates of bovine pituitary and hippocampus were analyzed to establish the nature of the immunoreactive material because they were two of the most strongly staining areas found in the immunohistochemical experiments.

Immunoblots of two-dimensional gels of pituitary extracts revealed that only a small group (Fig. 1D) of the large number of proteins (Fig. 1C) were immunoreactive. The immunoreactive proteins were similar to those found in the chromaffin granule lysate (Fig. 1B). A protein band in the pituitary extract, characteristically oblique, could be identified as chromogranin A (Fig. 1C). Amongst all the soluble proteins in the hippocampus (Fig. 2A), only two were immunoreactive. Mixing experiments showed that they comigrated, in both dimensions (Fig. 2B), with the two most intense bands in the adrenal extracts.

3.2.2. *Other brain areas*

Because the immunohistochemical experiments revealed an unexpectedly wide distribution of chromogranin-immunoreactivity, a range of brain regions were examined by the immunoblotting technique. Since most of the histochemical work was done using sheep tissues, extracts were taken from sheep brains and analyzed on one-dimensional gels. Soluble extracts from each of the areas were compared on the same gel with similar extracts of sheep adrenal medulla or the bovine chromaffin granule lysate.

All brain areas examined, except the lateral geniculate nucleus, showed immunoreactive bands. The pituitary extract stained the strongest and had several bands all similar to those in the adrenal medulla (Fig. 2C, lane 4). The intensity of staining of immunoblots did not parallel the intensity of immunohistochemical staining. For example, the CA3 region and the dentate gyrus of the hippocampal formation showed very strong staining in mossy fibre terminals (Fig. 5A). This staining was absent in the CA1 region although the majority of the pyramidal cells had weaker immunoreactivity in their Golgi apparatus (Fig. 6). On blots, these two areas gave bands with a similar intensity. In the lateral geniculate nucleus there were no bands on blots, and only very weak CHR-immunoreactivity in some of the cell bodies and in a few terminals. The major immunoreactive proteins in each of the brain areas had electrophoretic mobilities identical to the major components of the chromaffin granule lysate. The relative intensity of staining of these bands varied between different animals. For example, in one animal there was more of the higher molecular weight form in the dentate gyrus/CA3 region whilst in another animal there was more of the lower molecular weight material (Fig. 2C). These differences may reflect differences in autolysis that were impossible to control using material collected from a slaughterhouse. Smaller immunoreactive peptides, particularly one of M_r 50,000, were occasionally seen in certain areas. These bands were at the limit of sensitivity of the methods.

3.2.3. *Identity of antigenic determinants in brain and in the adrenal medulla*

Absorption tests were carried out by incubating

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Fig. 2. Identification of CHR-immunoreactivity in brain. The soluble proteins in an extract of bovine hippocampus (A and B) were analyzed by two-dimensional isoelectric focusing/electrophoresis (see Fig. 1). The gel was stained with Coomassie blue (A), and a blot with anti-chromogranin serum (B). Only two proteins reacted with anti-CHR serum, one similar to chromogranin A and one smaller protein. The soluble proteins from several areas of sheep brain were analyzed by immunoblots after one-dimensional SDS-gel electrophoresis and compared with both sheep and bovine adrenal proteins. All lanes except (1,2,3) contained approximately 100 μg of protein. Lanes contained the following samples: 1. Bovine chromaffin vesicle lysate (2 μg); 2. Bovine adrenal medulla soluble fraction (2 μg); 3. Sheep adrenal medulla soluble fraction (2 μg); 4. Sheep pituitary; 5. Hippocampus, dentate gyrus and CA3 region; 6. Hippocampus, CA1 region; 7. Cerebral cortex; 8. Globus pallidus; 9. Spinal cord, dorsal horn; 10. Spinal cord, ventral horn; 11. Amygdala; 12. Caudate nucleus; 13. Thalamus; 14. Hippocampus, as 5 but different animal; 15. As 6 but different animal; 16. Membrane pellet from hippocampus; 17. Lateral geniculate nucleus. Molecular weight calibrations were bovine serum albumin (M_r = 68,000) and bovine carbonic anhydrase (M_r = 29,000).

sections of ox, sheep and rat adrenal and sections of hippocampus of sheep and ox using post-embedding conditions.

The CHR-immunoreactivity in either the adrenal or brain was not affected by prior adsorption of the antisera with Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7, dynorphin$_{1-17}$, BAM22P or carbonic anhydrase.

High speed supernatant fractions from bovine hippocampus with protein concentrations of 15.7 or 13.5 mg/ml completely abolished staining in the bovine hippocampus and left only very faint staining in the sheep hippocampus when the antiserum was used at a final dilution of 1:800. The immunoreactivity gradually reappeared when the extract was diluted further. The same effect on the immunostaining of the chromaffin cells was also observed. Thus, following absorption of the CHR-antiserum with increasing concentrations of hippocampal protein extract the immunoreactivity was greatly attenuated but some staining was still present even at the highest protein concentration. All the immunoreactivity was abolished by prior absorption of the antiserum (1:400) with 100 or 20 μg/ml of the chromogranin containing lysate from the chromaffin granules, while at an antiserum dilution of 1:200 there was very weak staining in the adrenal only. At lower concentrations of (lysate) protein, the immunostaining gradually reappeared, and the intensity of the stain in the adrenal and hippocampus changed in parallel.

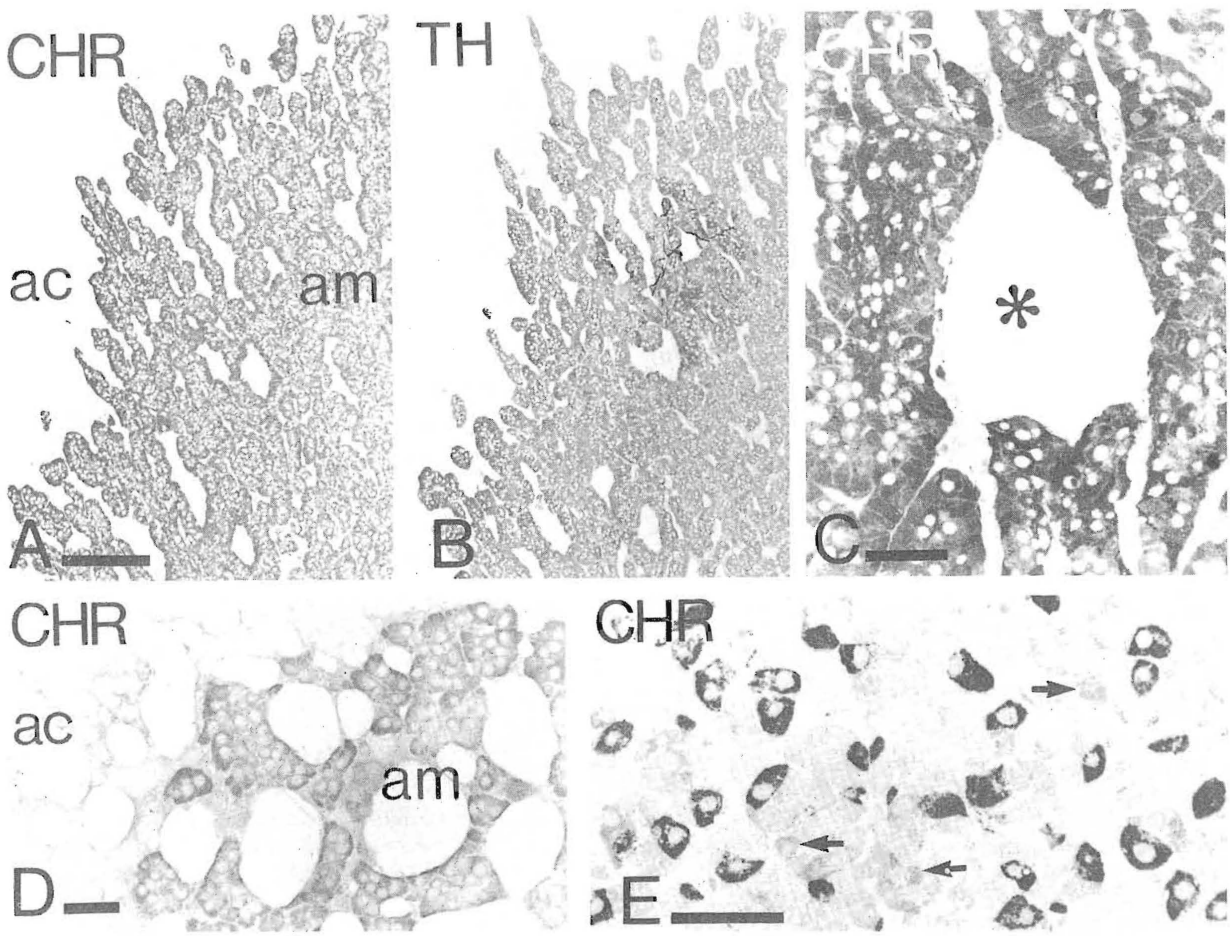


Fig. 3. Chromogranin (CHR; A, C, D) and tyrosine hydroxylase (TH, B) immunoreactivity in the adrenal glands of sheep (A, B), ox (C) and rat (D), as demonstrated on semithin sections by post-embedding immunohistochemistry. Chromogranin immunoreactivity is present only in the chromaffin cells of the medulla (am) and follows the same distribution as tyrosine hydroxylase. The chromaffin cells in C surround a sinusoid (asterisk). E. strongly (black) and weakly (arrows) chromogranin-immunoreactive cells in the anterior pituitary of a wether as demonstrated in a semithin section. ac, adrenal cortex; scales: A and B, same magnification, 200 μm; C–E, 50 μm.

These results demonstrate that the same antigens are recognized by the antisera in both brain and adrenal.

3.3. *Immunohistochemical localization of CHR-immunoreactivity*

3.3.1. *Adrenal gland*

Catecholamine producing chromaffin cells in sections of the adrenal medulla were identified by their immunoreactivity for TH when incubated under either pre- or post-embedding conditions. The antiserum to TH gave equally strong staining in adrenals from sheep (Fig. 3B), cattle, guinea pigs, rabbits and rats.

Serial sections of the adrenals were incubated to reveal CHR- and TH-immunoreactivity. Strong staining was obtained for CHR in adrenal chromaffin cells of the sheep (Fig. 3A) and ox (Fig. 3C) and, even at higher concentrations of antiserum, only weak immunoreactivity was observed in the rat (Fig. 3D), guinea pig and rabbit. In all species, the chromaffin cell nuclei, endothelial and connective tissue cells, and cells of the adrenal cortex did not show any immunoreactivity (Figs. 3A–D). In sections incubated under pre-embedding conditions the staining had a granular appearance in the cytoplasm of chromaffin cells.

The sheep and ox chromaffin cells were also strongly immunoreactive for Leu- and Met-enkephalin and Met-enkephalin-Arg^6-Phe^7 but were weakly reactive in the rat.

3.3.2. *Anterior pituitary*

Two populations of cells showed CHR-immunoreactivity in the anterior pituitary of the wether. One population stained as strongly as adrenal chromaffin cells and had a high cytoplasm to nucleus ratio (Fig. 3E). The other population was weakly immunoreactive and the cells were smaller. Many of the cells were not immunoreactive even after using a very high concentration of the anti-CHR serum. The same pattern of staining was obtained in the pituitary of the ewe.

3.3.3. *Pineal gland*

Noradrenergic sympathetic neurons are known to contain and release chromogranin[14,89]. Since the pineal gland receives its main innervation from noradrenergic neurons of the superior cervical ganglion[1], it was used to establish whether the CHR-immunoreactivity could be detected immunohistochemically in the terminal varicosities of such neurons. The noradrenergic axons, revealed by TH-immunoreactivity in the sheep pineal, ran in large fibre bundles (Fig. 4B). Axons from these bundles entered the parenchyma and branched out to form varicose fibres. Exactly the same pattern was observed in consecutive sections reacted with the CHR-antisera. The CHR-immunoreactivity was more pronounced in the varicosities and weaker in the axon bundles than the TH-immunoreactivity (Fig. 4A, B).

3.3.4. *Central nervous system*

3.3.4.1. *Light microscopic appearance of CHR-immunoreactivity*. Immunoreactivity could be demonstrated throughout the central nervous system of the sheep and cattle. Weak immunoreactivity was also detected in the brain of guinea pigs and rats using the pre-embedding technique.

The demonstration of CHR-immunoreactivity in these species required higher concentrations of the antiserum which resulted in high background staining. Therefore, the distribution of CHR-immunoreactivity was studied systematically in the sheep only.

Immunoreactivity due to CHR was localized to both neuronal perikarya and fibre networks. Stained perikarya fell into 3 broad classes: (1) most CHR-immunoreactive perikarya exhibited a perinuclear patchy network of staining often extending into the most proximal dendrites (Figs. 5B, D, 6A, 7D, 8). The distribution of this staining resembled the location of the Golgi apparatus; (2) in addition to this patchy staining, some groups of neurons exhibited a homogeneous staining throughout the cytoplasm (Figs. 10B, D, F, 11B); (3) some large neurons, such as motoneurons and the large neurons of the red nucleus, were characterized by staining in small patches throughout the cytoplasm (Fig. 8C). In each structure only one type of perikaryal staining was observed using either pre- or post-embedding techniques.

Immunostaining was also present in some well-defined fibre systems where it could be assumed to be in nerve terminals. In other, very heavily stained areas

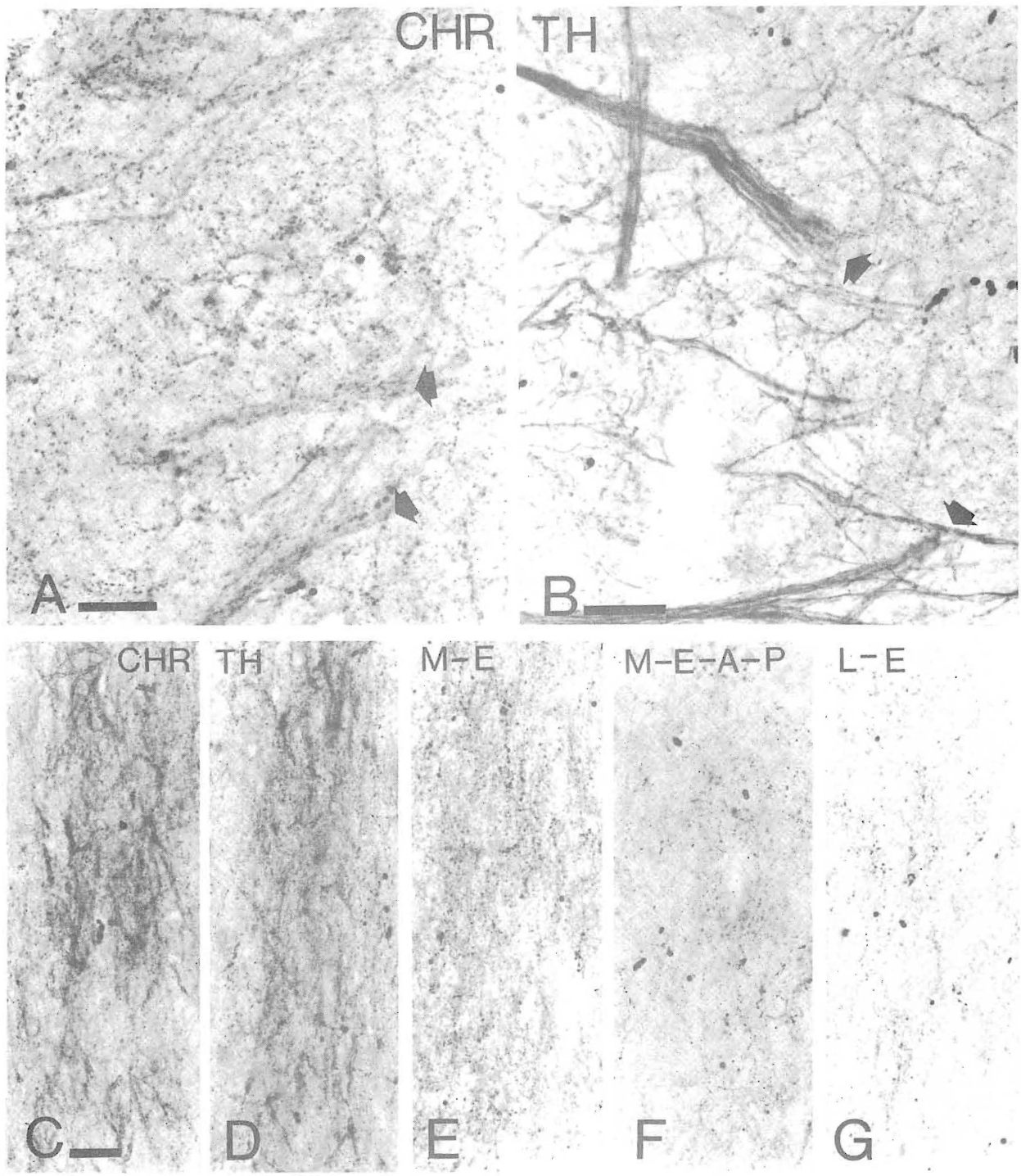


Fig. 4. Vibratome sections of the sheep pineal gland reacted for chromogranin (CHR, A) and tyrosine hydroxylase (TH, B). The immunoreactive fibre tracks (arrows) and punctate varicosities show similar distribution. C–G: vibratome sections of the sheep thalamic paraventricular nucleus reacted in pre-embedding immunocytochemistry. Chromogranin-immunoreactive terminals (C) similar to tyrosine hydroxylase immunoreactive ones (D) densely surround the dendrites of neurons. Met-enkephalin (E), met-enkephalin-Arg^6-Phe^7 (F), and Leu-enkephalin (G) immunoreactive terminals are fewer and more scattered in the neuropile. Scales: A–G, 50 μm; C–G, same magnification.

such as the globus pallidus or substantia nigra, the entire neuropil showed immunoreactivity (Figs. 7C, 9A, B). In most structures, however, small varicose fibres and punctae, probably representing nerve terminals, were observed. In some areas these surrounded neuronal perikarya, forming pericellular networks (triangles in Fig. 12).

3.3.4.2. *Electron microscopic localization of CHR-immunoreactivity*. Stained tissue sections were also examined in the electron microscope to determine the sub-cellular localization of the immunoreactive material. These studies were carried out using some of the most intensely stained areas of the sheep central nervous system, namely the hippocampus, the dorsal horn of the spinal cord and the caudate nucleus.

It was confirmed that the CHR-immunoreactive perinuclear network represented the Golgi apparatus (Fig. 6B–D). Large neurons of the caudate nucleus and the pyramidal cells of the CA3 region of the hippocampus were examined. The CHR-immunoreactivity was present in the lumen of Golgi saccules and, to a lesser extent, in vesicles. Usually, only the middle Golgi saccules were immunoreactive (Fig. 6C, D). Occasionally a few transitional elements between the rough endoplasmic reticulum and the cis side of the Golgi apparatus were also immunoreactive. As a result of these observations the network-like perikaryal staining will be referred to as 'Golgi apparatus'-type immunoreactivity in the following description.

The mossy fibre system of the hippocampus could be recognized as immunoreactive for CHR from its characteristic location (Fig. 5). When the mossy fibre terminals were studied using the pre-embedding technique, it was found that the CHR-immunoreactivity was mainly localized in the electron dense core of some of the large granulated vesicles. There was also immunoreactivity associated with intraterminal membranes, but to a lesser extent. The terminals formed asymmetric synaptic contacts with the apical dendritic shafts and complex spines of CA3 pyramidal cells (Fig. 5E–G). In the area of synaptic junctions, omega-shaped profiles containing electron dense cores were frequently observed. These profiles, probably representing large granulated vesicles undergoing exocytosis, were most frequently seen immediately adjacent to synaptic active zones (Fig. 5F–G). Some of the dense cores in the omega-shaped profiles showed immunoreactivity (Fig. 5G).

In the caudate nucleus of the sheep the immunoreactive varicose fibres formed symmetrical synaptic contacts and the immunoreactivity was also localized mainly in large granulated vesicles.

In the substantia gelatinosa of the dorsal horn of the spinal cord, most CHR-immunoreactive varicosities seen in the light microscope proved to be synaptic terminals or preterminal axons crowded with immunoreactive large granulated vesicles (Fig. 11B, C). The terminals formed asymmetrical synaptic contacts with dendrites (Fig. 11C) and perikarya. In contrast to the mossy fibres, most immunoreactive large granulated vesicles were situated at the periphery of the terminal away from the synaptic junction. The immunoreactive terminals were occasionally seen to be presynaptic to other vesicle containing profiles.

Since CHR-immunoreactive varicosities and punctae represented nerve terminals in all 3 areas, this term will also be used in the following description of results obtained with the light microscope.

3.3.4.3. *Comparison of the distribution of CHR-, TH-, DBH- and enkephalin- immunoreactivity*.

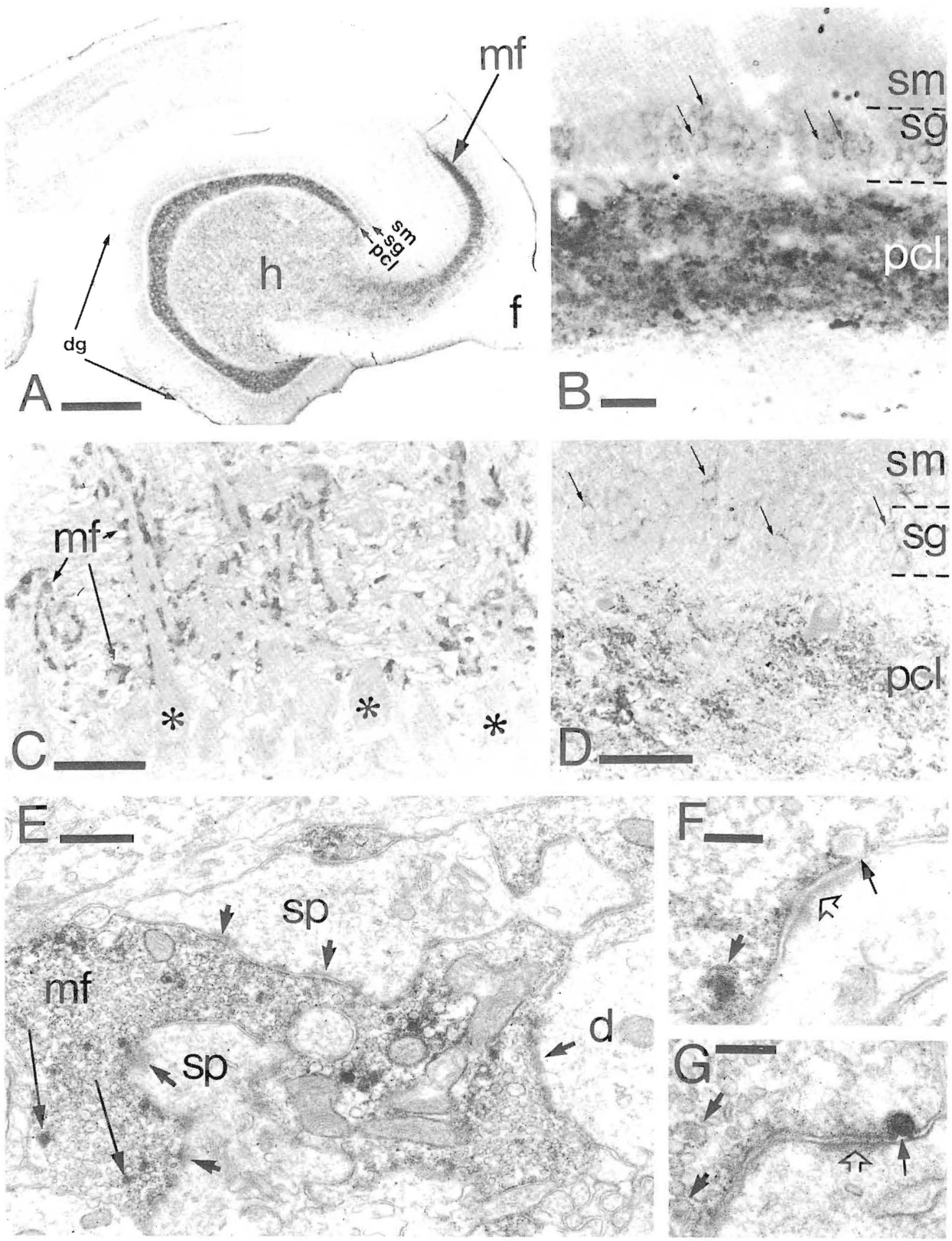
3.3.4.3.1. *Outline of description*.

The following description of the distribution of CHR in the brain of the sheep will progress from rostral to caudal (Fig. 12); where appropriate, observations made in bovine, rat and guinea pig brains will also be described. These experiments were designed to achieve an overall picture of the distribution of the CHR in the central nervous system. Areas such as the hypothalamus and thalamus, which have structurally and biochemically distinct subnuclei, will need to be examined in detail in a separate study.

Several markers (see Materials and Methods) of defined neuronal populations were examined in parallel with the CHR-immunoreactivity to determine whether they were present in the same neuronal population. The distribution of the different antigens will be compared only in structures where the staining suggested co-existence of the antigens, or where it was so different that particular neuronal systems could be excluded.

3.3.4.3.2. *Telencephalon*.

Cerebral cortex. Most pyramidal cell perikarya exhibited Golgi apparatus-type immunoreactivity for CHR (Fig. 8A), but the strongest reaction was in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neurons of layer V. The band of darkly stained layer V neurons could be followed throughout the neocortex. In the primary olfactory cortex, the neurons of layer II were the most strongly stained (Fig. 8B), and there was an abrupt shift in the strongly stained cells from layer II to layer V at the lateral border of the olfactory cortex. Sparse immunoreactive varicose fibres were found throughout the cortex from layer I to layer VI. Their distribution, course and density resembled that of the TH-immunoreactive catecholamine system, suggesting that CHR could be localized in the fibres containing catecholamines. The only exception to the rather uniform and sparse fibre distribution was the pyriform cortex where the outer portion of layer I had a dense and strongly stained fibre system which continued in the outermost layer of the olfactory tubercle.

Hippocampal formation. Pyramidal, granule and some non-pyramidal cells in sheep and cattle hippocampus showed Golgi apparatus-type immunoreactivity in their perikarya (Fig. 5B–D, 6A). The most strongly stained fibres were the axons and terminals of the granule cells (Fig. 5A). In both species there was a well-delineated band of terminal staining in the polymorphic cell layer (Fig. 5A–D) followed by sparser staining in the hilus and by a strong band in the CA3 region where the mossy terminals make synapses with the apical dendrites. In addition there was a less conspicuous, narrow band of stained fibres and terminals in the stratum oriens of the CA2–CA1 regions immediately bordering the alveus. Occasional CHR-immunoreactive fibres were encountered in all regions and layers of the hippocampal formation including the subiculum. In the rat and guinea pig hippocampus, the mossy fibre system showed only very weak CHR-immunoreactivity.

The distribution of TH did not resemble that of CHR. Branching axons with varicose terminal segments were present in all areas and layers, but the mossy fibre system and fibres near the alveus were not TH-positive. The distributions of Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 immunoreactive varicose fibres were similar to each other and were confined to sparse, scattered, branching fibres in all layers and areas. None of the antisera to opiate peptides used in the present study reacted with the mossy fibre system.

Basal ganglia. The large neurons in the caudate nucleus and putamen exhibited very strong Golgi apparatus-type CHR-immunoreactivity (Fig. 7D) and CHR-positive neurons of similar size were also distributed in and around the the globus pallidus. Only some of the medium sized neurons which make up the majority of the cells in the caudate and putamen area, showed weak CHR-immunoreactivity and some were immunoreactive for Met-enkephalin. The nucleus accumbens contained few strongly CHR-stained cells, but they were more numerous in and around the islands of Calleja. The olfactory tubercle (as delineated by its strong AChE activity) contained no CHR-immunoreactive perikarya.

Immunoreactive varicose fibres and immunoreactive terminals were seen throughout the basal ganglia (see 3.3.4.2.). The caudate nucleus and putamen contained a moderate density of terminals which were distributed unevenly (Fig. 12). There were weakly immunoreactive patches, accompanying myelinated axon bundles heading to join the internal capsule. In the area including the nucleus accumbens, the islands of Calleja and the olfactory tubercle, a complex mosaic of strongly, moderately or, weakly stained, but sharply delineated patches, was

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Fig. 5. Chromogranin immunoreactivity in the hippocampal formation. A: vibratome section of the hippocampus of sheep showing strong immunoreactivity in granule cell terminals which are distributed in the polymorphic cell layer (pcl) of the dentate gyrus (dg), in the hilus (h) and in the mossy fibre system (mf). B: higher magnification photograph of the same section as in A. In the dentate gyrus, immunoreactivity is present in the granule cells (arrows) and in their terminals in the polymorphic cell layer (pcl). C: semithin section of the CA3 region of sheep hippocampus showing immunoreactivity in the boutons of mossy fibres (mf) terminating on the apical dendrites of pyramidal cells (asterisks). D: semithin section of the dentate gyrus of ox showing immunoreactivity in granule cell somata (arrows) and in their terminals in the polymorphic cell layer (pcl). E–G: electron micrographs of immunoreactive mossy fibre boutons (mf) in the CA3 region of the sheep. E: immunoreactivity is mainly localized to large granulated vesicles (long arrows). The boutons make numerous synaptic contacts (short arrows) with spines (sp) and a dendrite (d) of pyramidal cells. F. and G: next to the synaptic specialisation (open arrow) omega-shaped profiles, containing either immunoreactive (thin arrow in G) or non-immunoreactive (thin arrow in F) dense cores, are frequently encountered. The mossy terminals contain both immunoreactive (thick arrow in F) and non-immunoreactive (thick arrows in G) large granulated vesicles. sg, stratum granulosum; sm, stratum moleculare. Scales: A, 1 mm; B–D, 50 μm; E, 0.5 μm; F and G, 0.2 μm.

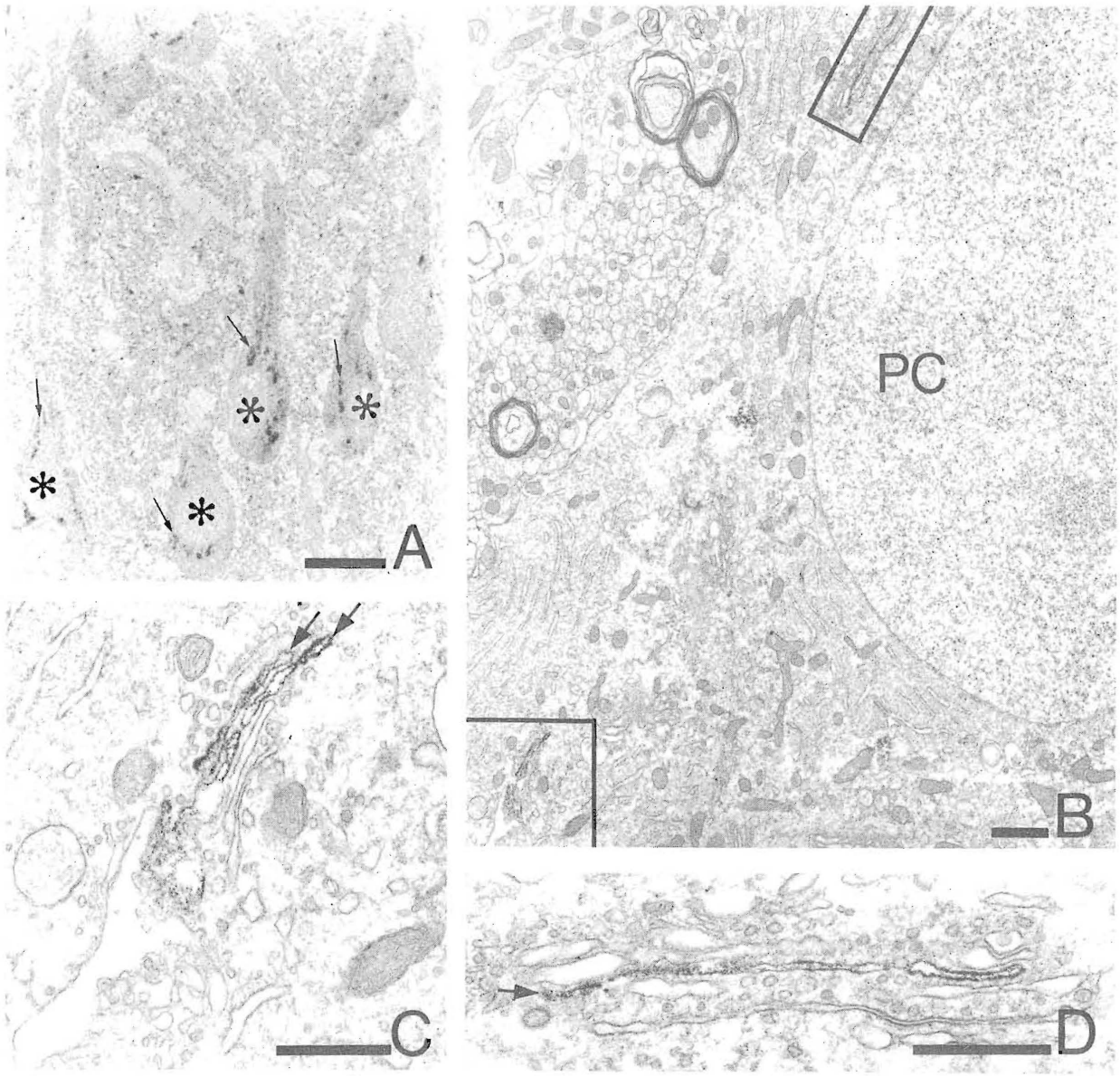


Fig. 6. Chromogranin immunoreactivity in the Golgi apparatus. A: semithin section of the CA1 region of ox hippocampus. Immunoreactivity is localized in small perinuclear patches resembling the Golgi apparatus in the pyramidal neurons. B: electron micrograph of a pyramidal cell (PC) in the CA3 region of the sheep hippocampus. Framed areas contain chromogranin immunoreactive Golgi apparatus and are shown at higher magnification in C and D. C and D: immunoreactivity is localized within some of the Golgi saccules (arrows). Scales: A, 20 μm; B, 1 μm; C and D, 0.5 μm.

observed (Fig. 7B). By incubating serial sections to reveal the enkephalin immunoreactivity, it could be demonstrated that the mosaic pattern of CHR-immunoreactivity matched the pattern of the opiate peptides. The weakly stained CHR patches were in the same position but were usually broader for the enkephalins. The pattern obtained for the 3 enkephalins was identical.

The intensity of staining for AChE activity, known to have an uneven distribution in components of the neostriatal complex[36], also correlated well with the level of CHR-immunoreactivity (Fig. 7A, B).

Fibres and terminals in the globus pallidus and entopeduncular nucleus were among the most strongly

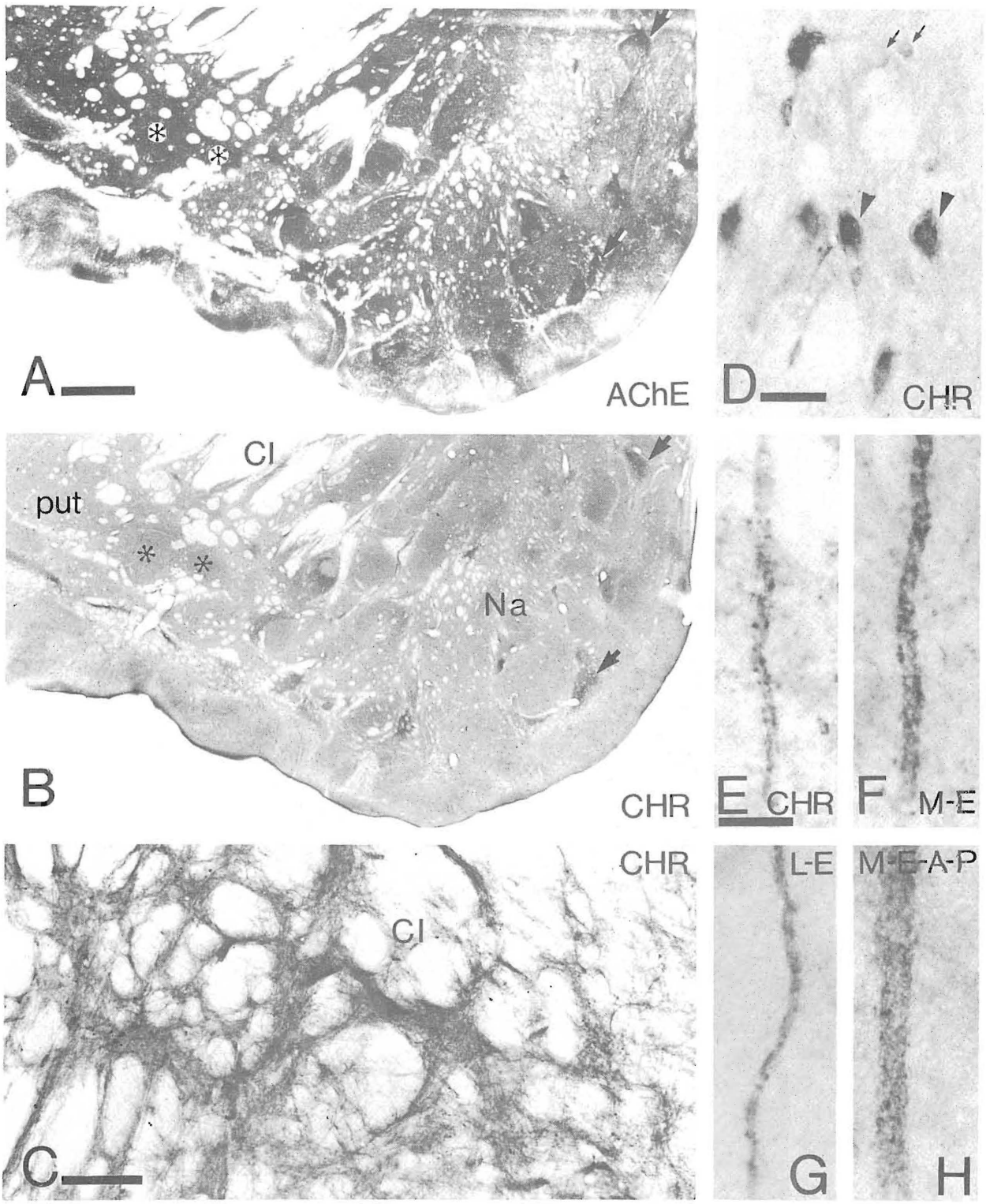


Fig. 7. A and B: ventral forebrain of the sheep reacted to reveal acetylcholinesterase (AChE) enzyme activity (A) and chromogranin immunoreactivity (CHR, B). Both show a patchy distribution in the nucleus accumbens (Na) olfactory tubercle and in the putamen (put). Areas showing higher AChE activity (asterisks, arrows) stain stronger for chromogranin immunoreactivity. Fibre bundles of the capsula interna (CI) and other myelinated axon pathways show up as pale areas. C: chromogranin immunoreactivity in the globus pallidus of the sheep. Immunoreactive terminals densely surround dendrites distributed among the fibre bundles of the internal capsule (CI). D: strong chromogranin immunoreactivity in the large neurons (arrowheads) of the caudate nucleus of the sheep. Small and medium size neurons (arrows) are either weakly immunoreactive or not immunoreactive at all. E–H: dendrites in the ventral caudate nucleus of the sheep ensheathed by boutons immunoreactive for chromogranin (E), Met-enkephalin (F), Leu-enkephalin (G) or Met-enkephalin-Arg^6-Phe^7 (H). Scales: A and B, 1 mm; C, 200 μm; D, 50 μm; E–H, same magnification, 20 μm.

CHR-immunoreactive elements in the brain (Fig. 7C). Cell bodies and thick dendrites were completely surrounded by CHR-immunoreactive terminals. Neurons ensheathed in this way penetrated the ventral neostriatum where their dendrites were very conspicuous (Fig. 7E).

This neuronal system also extends rostrally, forming the ventral pallidum and groups of CHR-fibres ensheathed neurons around the islands of Calleja. The pattern of CHR immunoreactivity matched exactly that of the 3 opiate peptides (Fig. 7E–H). This distribution of CHR-immunoreactivity in the sheep was the same as that in ox. Thus, the distribution of immunoreactive perikarya was similar, and the globus pallidus and entopeduncular nucleus contained heavily immunoreactive CHR-positive fibres and

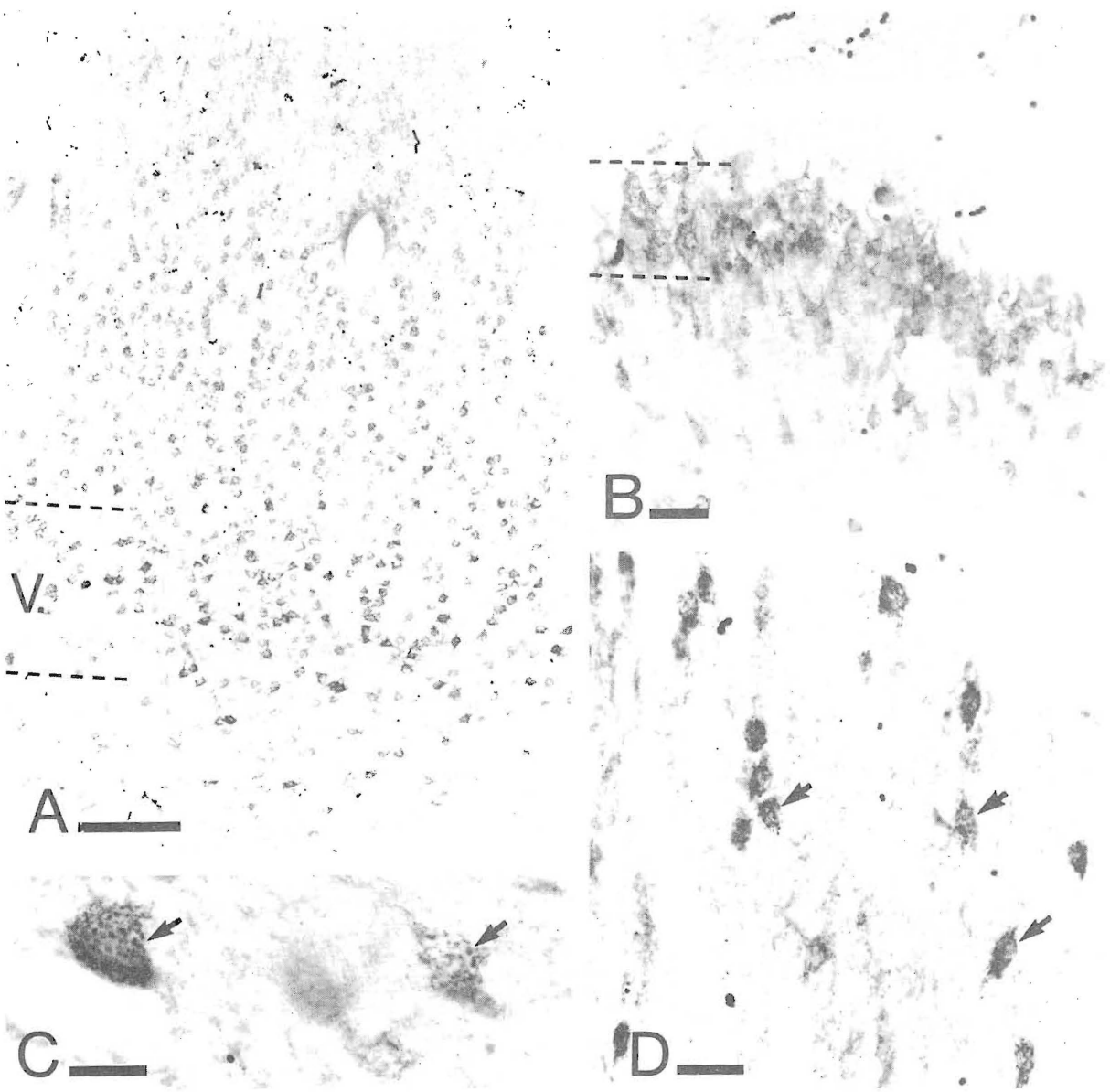


Fig. 8. Chromogranin immunoreactivity is localized in perinuclear halo within neuronal perikarya in the gyrus orbitalis of the frontal cortex (A), in neurons of the olfactory cortex (B), in motoneurons (arrows) of the spinal cord (C) and in neurons (arrows) of the vertical arm of the nucleus of the diagonal band (D). Note that in the neocortex the pyramidal cells of layer V. are the most strongly immunoreactive, while the same is true in the olfactory cortex for neurons of layer II (dashed lines) Scales: A, 200 μm; B–D, 50 μm.

terminals.

Claustrum. Neurons showed weak or, rarely, strong Golgi apparatus-type immunoreactivity.

Septum. The medial septum contained strongly immunoreactive neurons and this group was continuous with strongly immunoreactive neurons in both arms of the nucleus of the diagonal band. Similar strongly stained neurons, showing Golgi apparatus-type immunoreactivity, were scattered further caudally along the ventral forebrain and continued among the neurons of the globus pallidus. This distribution paralleled the strongly AChE-reactive cells in the basal forebrain. In contrast, the lateral septum contained no immunoreactive perikarya, but most neurons were surrounded by CHR-immunoreactive terminals. There were only a few terminals in the medial septum. Enkephalin-immunoreactive terminals had a similar distribution in the septum.

Bed nucleus of the stria terminalis. There were few immunoreactive neurons, but there was a dense CHR-immunoreactive fibre and terminal network.

Amygdala. Strongly CHR-positive neurons exhib-

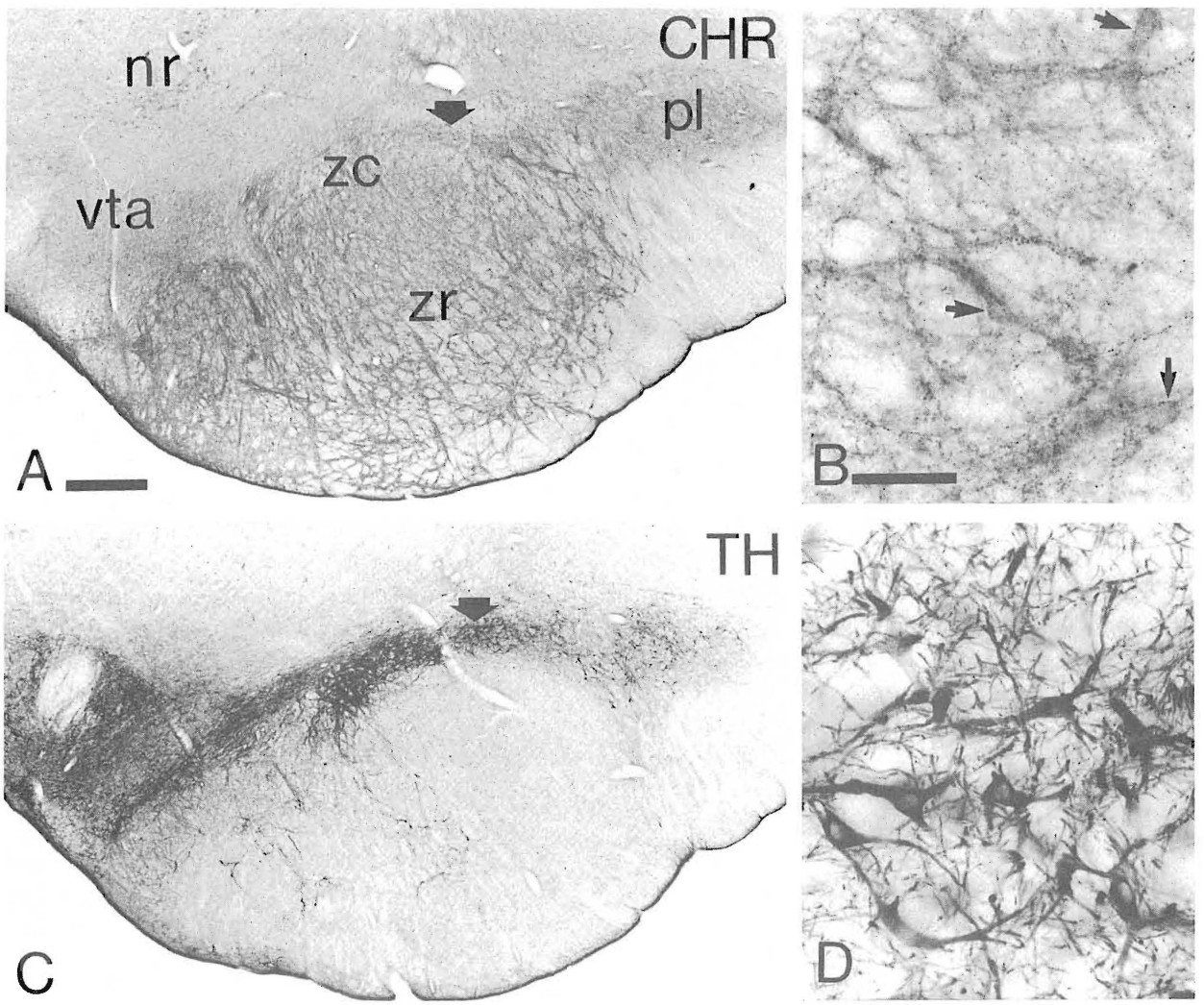


Fig. 9. Chromogranin (A and B) and tyrosine hydroxylase (C and D) immunoreactivity in the substantia nigra region of sheep as demonstrated by pre-embedding immunohistochemistry. Dense chromogranin fibre network is present in the zona compacta (zc), zona reticulata (zr) and pars lateralis (pl). The ventral tegmental area (vta) has fewer fibres and only the cell bodies are immunoreactive in the nucleus ruber (nr). Large arrows in A and C mark regions shown in B and D, respectively. The chromogranin immunoreactive boutons in B surround the zona compacta cells (arrows) most of which are dopaminergic as seen in D. Scales: A and C, same magnification, 1 mm; B and D, same magnification, 100 μm.

iting Golgi apparatus-type staining were present in the central and basolateral nuclei, while neurons of the lateral basomedial and basolateral ventral nuclei showed weak immunoreactivity. No immunoreactivity could be demonstrated in neurons of the medial nucleus. A very dense CHR-positive terminal network was found in the central nucleus with fewer fibres in the medial, basomedial and cortical nuclei. The central nucleus also stained very strongly for the 3 enkephalins and for TH. In the medial, basomedial and cortical nuclei, enkephalin-immunoreactive fibres were sparser than CHR-fibres.

3.3.4.3.3. *Diencephalon.*

Thalamus. Neurons in most nuclei showed very weak Golgi apparatus-type CHR-immunoreactivity and only occasional stained fibres were seen. Therefore, the nuclei were not delineated in Fig. 12. An exception was the thalamic reticular nucleus which contained some strongly stained neurons and had a moderately stained fibre network throughout the neuropil. This fibre staining did not correlate with TH or enkephalin immunoreactivity. The paraventricular thalamic nucleus and its continuation in patches along the midline showed a very strongly stained CHR-fibre network. The proximal dendrites of neurons, and to a lesser extent the perikarya, were completely surrounded by CHR-immunoreactive terminals (Fig. 4C). This pattern closely resembled that of TH- (Fig. 4D) and DBH-immunoreactivity. This nucleus of the thalamus also showed the highest density of enkephalin-immunoreactive terminals; Met-enkephalin staining was especially strong. The terminals appeared to be more scattered than those positive for CHR.

Hypothalamus. Only weakly immunoreactive neurons were seen. Many of the magnocellular neurons in the paraventricular and supraoptic nuclei exhibited Golgi apparatus-type staining as well as more homogeneous staining throughout the perikarya. Many terminals in the neural lobe of the pituitary were strongly CHR-immunoreactive. The hypothalamus was very rich in varicose fibres and terminals immunoreactive for CHR. The densest areas were the median eminence, the periventricular region, the nucleus arcuatus and the supraoptic nucleus. A smaller, but still significant, number of fibres and terminals were seen in the paraventricular nucleus. Additional fibres were also observed throughout the hypothalamus but particularly in the ventromedial nucleus, in the lateral hypothalamus and in the area of the A13 catecholaminergic cell group over its full rostro-caudal extent. The fibres were often surrounding neuronal perikarya and proximal dendrites. The distribution of the catecholamine containing neurons was ascertained by TH-immunohistochemistry.

Both TH- and enkephalin-immunoreactive fibre systems were present in the areas of CHR-immunoreactive terminals, but the distribution of the CHR did not exclusively correspond to either. In the arcuate nucleus and in the median eminence, the distribution of CHR-immunoreactive terminals was different from that of the enkephalins and TH.

Zona incerta. Scattered immunoreactive neurons and only occasional terminals were seen.

3.3.4.3.4. *Mesencephalon.*

Strong, patchy staining was observed in neurons of the nucleus ruber, and oculomotor nuclei, while Golgi apparatus-type CHR-immunoreactivity was present in large cells of pars reticulata and pars lateralis of the substantia nigra, in neurons of the interstitial nucleus of Cajal, nucleus of Darkschewitsch and in the accessory oculomotor nuclei. Weak Golgi apparatus type-immunoreactivity was observed in some scattered cells of the mesencephalic reticular formation.

The areas where the neuropil was most heavily stained were the pars reticulata and lateralis of the substantia nigra (Fig. 9A). The immunoreactivity, like that in the globus pallidus, seemed to surround the dendrites completely. Heavy staining of a different type was found in the dopaminergic cell regions of the zona compacta, where varicose fibres often surrounded neuronal perikarya (Fig. 9). The regions of the ventral tegmental area containing dopamine cells had fewer CHR-immunoreactive fibres. The periaqueductal grey matter contained fibres and terminals throughout and some individual neurons were very heavily surrounded by terminals. The superior colliculus, the inferior colliculus, the medial geniculate nucleus and the mesencephalic reticular formation contained few immunoreactive fibres. Terminals were present throughout the interpeduncular nucleus.

Neither TH- nor enkephalin-immunoreactive fibre systems correlated completely with the CHR. The pars reticulata of the substantia nigra contained no

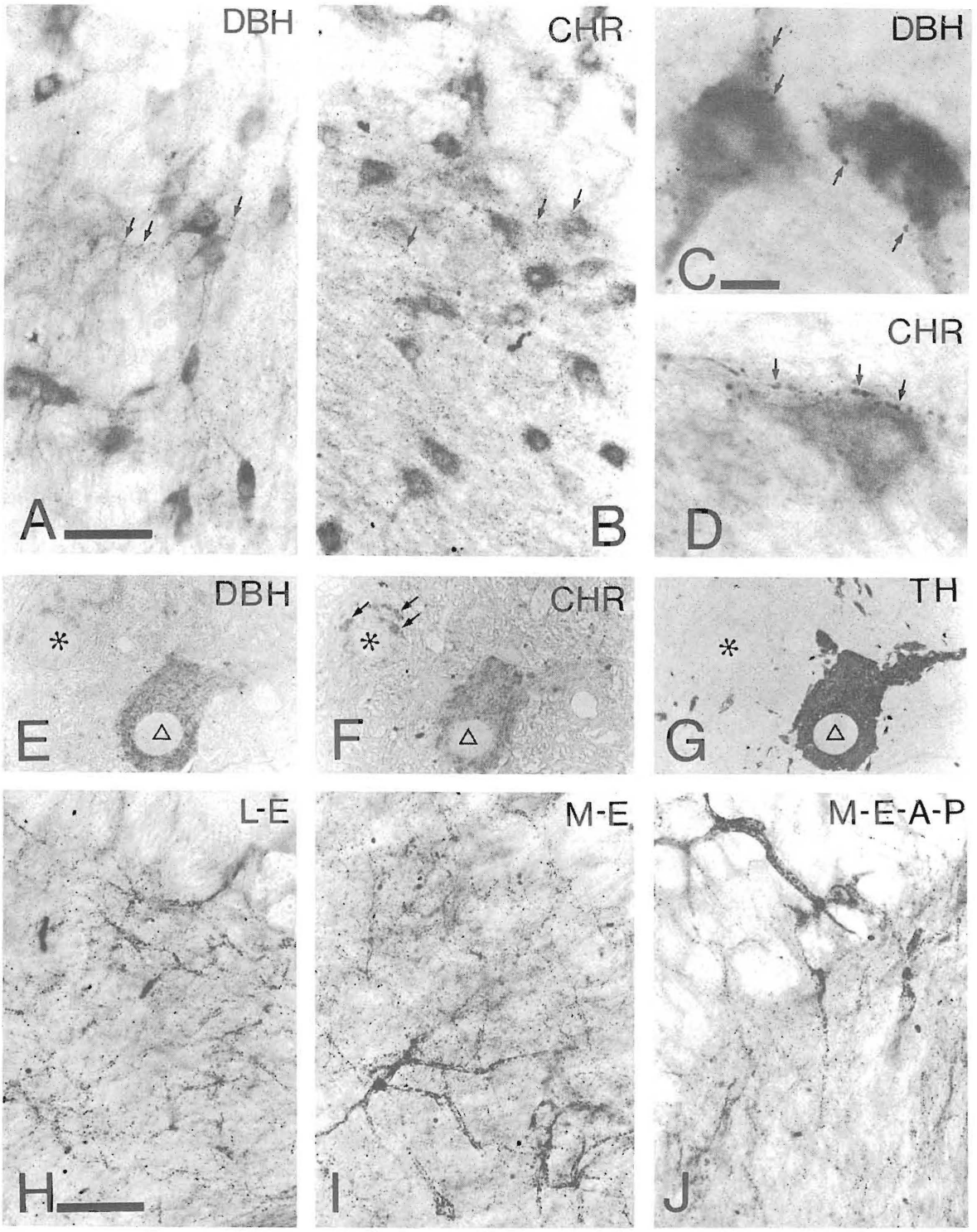


Fig. 10. Locus coeruleus of the sheep, reacted by pre-embedding immunohistochemistry of vibratome sections (A–D and H–J) and post-embedding immunohistochemistry of semithin sections. A and B: dopamine β-hydroxylase immunoreactivity (A) and chromogranin immunoreactivity (B) show a similar distribution localized to neuronal perikarya and varicose fibres (arrows). C and D: some of the varicosities are in apparent contact with the cells. E–G: serial sections of two neurons, one of which (triangle) is noradrenergic as shown by immunoreactivity for dopamine β-hydroxylase (E) and tyrosine hydroxylase (G). Chromogranin immunoreactivity (F) is also present in this neuron throughout the cytoplasm, but only in the Golgi apparatus (arrows) of the non-catecholaminergic neuron (asterisk). H–J: Leu-enkephalin-(H), Met-enkephalin-(I) and Met-enkephalin-Arg^6-Phe^7-(J) immunoreactive terminals show a distribution different from those shown in (A) and (B) and completely surround the perikarya and dendrites of neurons. Scales: A, B and H–J, same magnification, 100 μm; C–G, same magnification, 20 μm.

TH- or enkephalin-immunoreactive fibres. In the pars compacta on the other hand, immunoreactive fibres could be demonstrated with all 3 anti-enkephalin antisera, and the pattern and density of Met-enkephalin immunoreactivity resembled that of CHR. The same was true for the ventral tegmental area. In the periaqueductal grey and interpeduncular nucleus the TH-immunoreactive fibres were clearly different from those staining for CHR, but both areas were rich in enkephalin immunoreactive fibres and terminals with a distribution similar to the CHR-immunoreactivity.

3.3.4.3.5. *Pons and dorsal tegmentum.*

Locus coeruleus area. Strong CHR-immunoreactivity was present in neurons in all the areas of the A4, A6 and A7 catecholaminergic cell groups. CHR-, TH- and DBH-immunoreactive neurons were present in the locus coeruleus and subcoeruleus, in the parabrachial nuclei and scattered laterally throughout the dorsolateral tegmentum ending at the lateral lemniscus (Figs. 10A, B, 12). Using serial semithin sections of the same neuron, it could be demonstrated that immunoreactivity due to CHR, DBH and TH was present in the same neurons (Fig. 10E–G). The CHR-immunoreactivity in the cytoplasm of noradrenergic neurons was homogeneously distributed and there was also Golgi apparatus-type staining (Fig. 10D, F). Non-catecholaminergic neurons in the same area showed only the Golgi apparatus-type reaction. The area of the catecholamine producing cells was also rich in CHR-immunoreactive terminals many of them contacting CHR-positive neurons (Fig. 10D). Their distribution correlated well with DBH- and TH-positive terminals but more CHR-terminals were seen in the parabrachial nuclei. Although the area was very rich in enkephalin-immunoreactive terminals, their distribution was different since they completely ensheathed the perikarya and dendrites of neurons in the area of the catecholamine producing cells. The parabrachial nuclei contained few cells densely surrounded by enkephalin-immunoreactivity but also exhibited a dense network of immunoreactive fibres and terminals.

Other areas. Weak CHR-immunoreactivity, mainly of the Golgi apparatus-type, was present in neurons of the trochlear nucleus, the dorsal tegmental nucleus, the pontine nuclei, the nucleus of the lateral lemniscus and in the scattered neurons of the reticular formation. Moderate fibre- and terminal-staining was present in the dorsal and medial raphe nuclei, in the dorsal and dorso-lateral tegmental nuclei, in the pontine nuclei and in an area within the nucleus of the lateral lemniscus. One small, well-delineated area in the dorsal tegmental nucleus was strongly immunoreactive.

3.3.4.3.6. *Cerebellum.*

In the cerebellar cortex, only the Golgi cells showed weak Golgi apparatus-type immunoreactivity. Varicose fibres and terminals immunoreactive for CHR were present in the granule cell layers as well as among the Purkinje cells. The densest fibre network was in the molecular layer. The density and distribution of CHR-positive fibres correlated well with the TH-immunoreactivity. Groups of terminals immunoreactive for the enkephalins were found in the granular layer and they may correspond to the terminals of the Golgi cells. Very strong Golgi apparatus-type CHR-immunoreactivity was seen in neurons of the deep cerebellar nuclei, and there was also a sparse but strongly stained varicose fibre network. This latter immunoreactivity had a similar distribution to TH.

3.3.4.3.7. *Medulla oblongata and spinal cord.*

Cell bodies. Motoneurons of the abducens, facial, hypoglossal nuclei and the ventral horn of the spinal cord showed strong, patchy immunoreactivity, while neurons in the dorsal motor nucleus of the vagus were not immunoreactive. Strong immunoreactivity was also found in cells of the lateral reticular nucleus and in cells of the A1 and C1 catecholamine cell groups. No immunoreactivity was present in perikarya in the nucleus of the solitary tract and area postrema, which contained numerous TH-positive neurons. Strongly immunoreactive cells were scattered in the reticular formation. Small homogeneously stained neurons could be found infrequently in the substantia gelatinosa of both the spinal trigeminal nucleus and the dorsal horn of the spinal cord (Fig. 11B). Weak Golgi apparatus-type immunoreactivity was present in the neurons of the cuneate and external cuneate nuclei in deeper parts of the spinal trigeminal nucleus and in the vestibular nuclei.

Fibres and terminals. The densest immunoreactive terminal networks were found in the nucleus of the solitary tract, the area postrema, with less but still significant staining in the dorsal motor nucleus of the

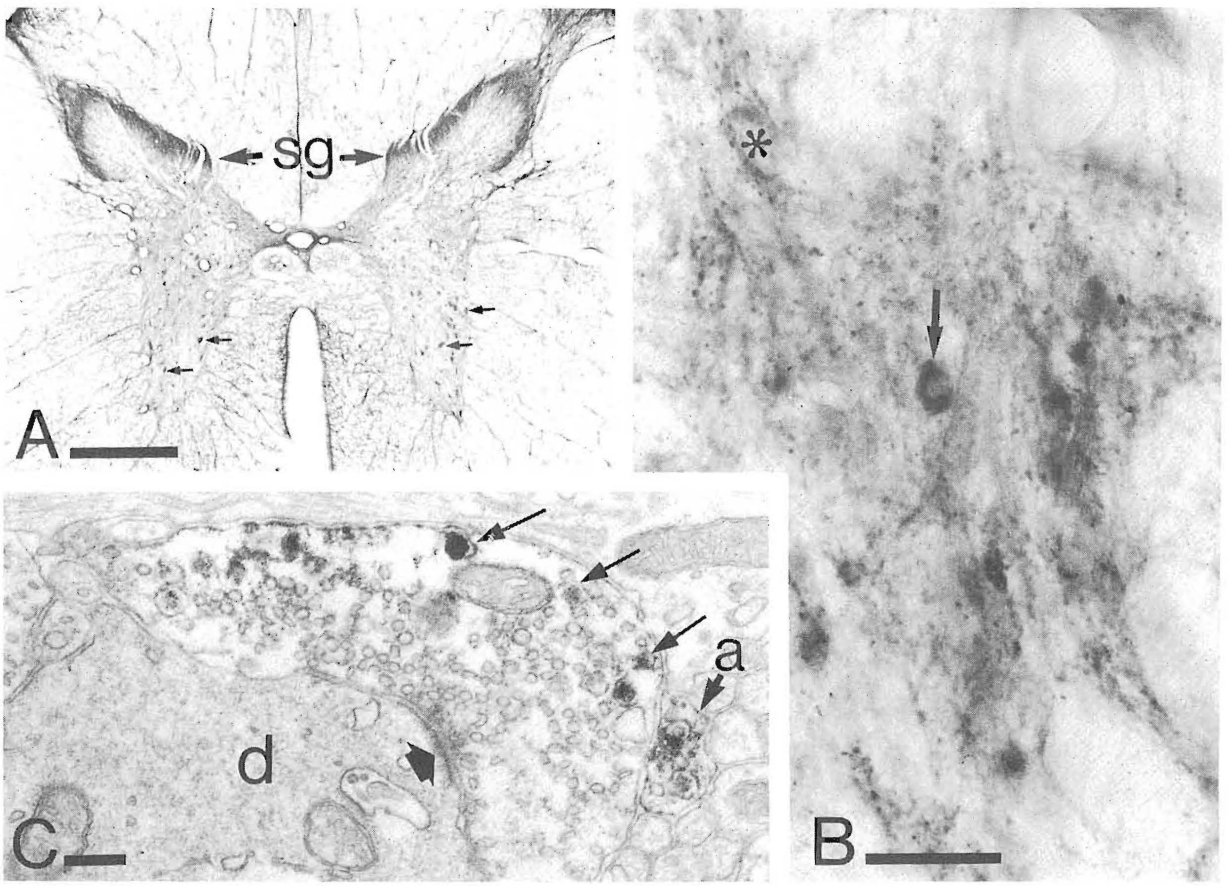
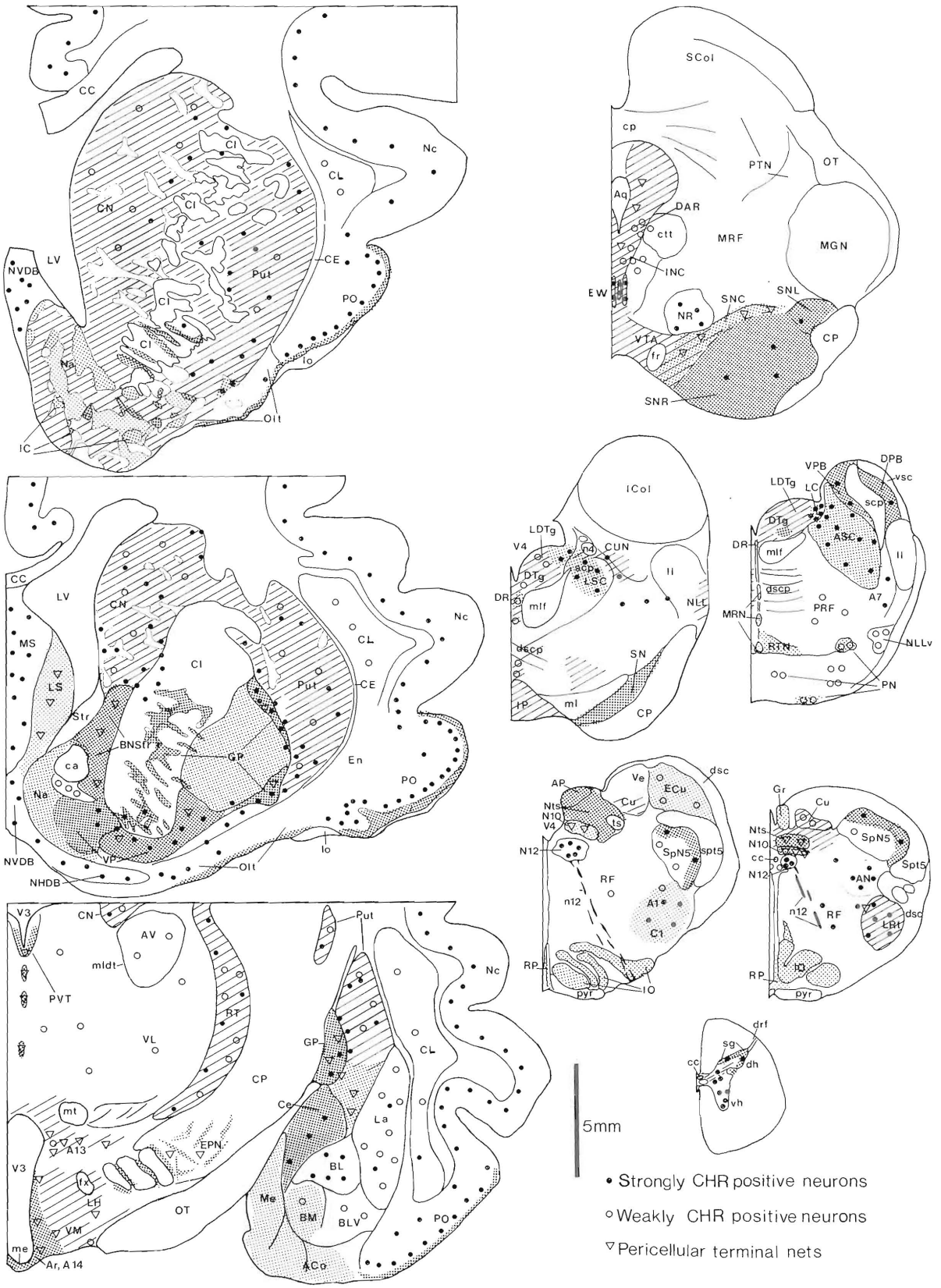


Fig. 11. A: vibratome section of the sheep cervical spinal cord showing strong chromogranin immunoreactivity in the substantia gelatinosa (sg) and in motoneurons (small arrows). B: in the substantia gelatinosa, immunoreactivity is present in fibres and boutons some of which surround non-immunoreactive neurons (asterisk). Immunoreactive perikarya (arrow) are also present. C: electron micrograph of an immunoreactive bouton establishing asymmetrical synaptic contact (thick arrow) with a dendrite (d). Immunoreactivity is mainly localized to large granulated vesicles (thin arrows) and is also present in a preterminal axon (a). Scales: A, 1 mm; B, 50 μm; C, 0.2 μm.

vagus. In the latter, the terminals surrounded the perikarya of the large neurons, similar to TH and enkephalin immunoreactive terminals. The area postrema and the nucleus of the solitary tract were also very rich in TH, and enkephalin-immunoreactive terminals whose density and distribution were similar to the CHR-immunoreactivity.

The other strongly CHR-stained terminal network was localized in the substantia gelatinosa of both the spinal trigeminal nucleus and the dorsal horn of the spinal cord (Fig. 11B). (See 3.3.4.2 for electron microscopic data). Some of the fibres surrounded small neurons (Fig. 11B). The areas containing CHR-immunoreactive fibres were also rich in enkephalin-positive fibres with a similar pattern and density. On the other hand, there were fewer TH-immunoreactive terminals. In the spinal cord, an area around and lateral to the central canal also contained numerous CHR-immunoreactive terminals. Single varicose fibres were also seen among the motoneurons of the ventral horn. Enkephalin-immunoreactivity had a similar distribution at these latter two sites.

The inferior olivary nucleus contained CHR-immunoreactive terminals throughout, but there were no enkephalin-immunoreactive fibres and few TH-positive terminals. In contrast, the density of CHR-immunoreactive terminals in the A1 and C1 catecholamine cell groups correlated well with enkephalin-immunoreactivity. A dense CHR-fibre network was also found in the gracile nucleus and fibres could be


CC
Cl
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MRF
MGN
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SNC
SNL
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LDTg
V4
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CUN
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II
NLL
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VPB
DPB
vsc
LC
ASC
A7
PRF
MRN
RTN
NLLv
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LS
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Me
BM
BLV
ACo
AP
Ve
dsc
ECu
Cu
Nts
N10
ts
N12
SpN5
spt5
RF
n12
A1
C1
RP
pyr
IO
Gr
AN
LRt
drf
sg
cc
dh
vh
5mm
• Strongly CHR positive neurons
○ Weakly CHR positive neurons
▽ Pericellular terminal nets

ABBREVIATIONS REFERRED TO IN FIG. 12

A Co	cortical amygdaloid nucleus
AN	ambiguous nucleus
AP	area postrema
Aq	aqueductus cerebri
Ar	arcuate nucleus
ASC	area subcoeruleus
AV	anterior ventral thalamic nucleus
A1	catecholamine cell groups
A7	catecholamine cell groups
A13	catecholamine cell groups
A14	catecholamine cell groups
BL	basolateral amygdaloid nucleus
BLV	basolateral ventral amygdaloid nucleus
BM	basomedial amygdaloid nucleus
BNStr	bed nucleus of the stria terminalis
ca	anterior commissure
cc	central canal
CC	corpus callosum
CE	capsula externa
CI	capsula interna
CL	claustrum
CN	caudate nucleus
cp	posterior commissure
CP	cerebral peduncle
ctt	central tegmental tract
Cu	cuneate nucleus
CUN	cuneiform nucleus
Cl	catecholamine cell group
DAR	nucleus of Darkschewitsch
dh	dorsal horn
DPB	dorsal parabrachial nucleus
DR	dorsal raphe nucleus
drf	dorsal root fibres
dsc	dorsal spinocerebellar tract
dscp	decussation of the superior cerebellar peduncle
DTg	dorsal tegmental nucleus
ECu	external cuneate nucleus
En	endopiriform nucleus
EPN	entopeduncular nucleus
EW	accessory oculomotor nucleus
fr	fasciculus retroflexus
fx	fornix
GP	globus pallidus
Gr	gracile nucleus
IC	islands of Calleja
ICol	inferior colliculus
INC	interstitial nucleus of Cajal
IO	inferior olive
IP	interpeduncular nucleus
La	lateral amygdaloid nucleus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamus
ll	lateral lemniscus
lo	lateral olfactory tract
LRt	lateral reticular nucleus
LS	lateral septum
LSC	locus subcoeruleus
LV	lateral ventricle
me	median eminence
Me	medial amygdaloid nucleus
ml	medial lemniscus
mldt	medullary lamina of the dorsal thalamus
mlf	medial longitudinal fasciculus
MGN	medial geniculate nucleus
MRN	medial raphe nucleus
MRF	mesencephalic reticular formation
MS	medial septum
mt	mamillo-thalamic tract
Na	nucleus accumbens
Nc	neocortex
NHDB	nucleus of the horizontal arm of the diagonal band
NLL	nucleus of the lateral lemniscus
NR	nucleus ruber
Nts	nucleus of the solitary tract
NVDB	nucleus of the vertical arm of the diagonal band
n4	tract of trochlear nerve
N10	dorsal motor nucleus of vagus
n12	tract of hypoglossal nucleus
N12	hypoglossal nucleus
Olt	olfactory tubercle
OT	optic tract
PN	pontine nuclei
PO	pyriform cortex
PRF	pontine reticular formation
PTN	pretectal nuclei
Put	putamen
PVT	paraventricular thalamic nucleus
pyr	pyramid
RF	reticular formation
RP	raphe pallidus
RT	reticular thalamic nucleus
RTN	reticulotegmental nerve of pons
SCol	superior colliculus
scp	superior cerebellar peduncle
sg	substantia gelatinosa
SN	substantia nigra
SNC	substantia nigra pars compacta
SNL	substantia nigra pars lateralis
SNR	substantia nigra pars reticulata
SpN5	spinal trigeminal nucleus
Spt5	spinal tract of trigeminal nerve
Str	stria terminalis
vh	ventral horn
Ve	vestibular nucleus
VL	ventrolateral thalamic nucleus
VM	ventromedial nerve of hypothalamus
VP	ventral pallidum
VPB	ventral parabrachial nerve
vsc	ventral spinocerebellar tract
VTA	ventral tegmental area
V3	third ventricle
V4	fourth ventricle

←

Fig. 12. Distribution of chromogranin (CHR) immunoreactivity in neuronal perikarya (dots, circles), fibres and terminals (stippled, hatched areas) in frontal sections of the sheep brain. Hatched areas contained a low density of fibres and the density of stippling corresponds to the density of immunoreactivity in the neuropil. Areas not labeled, contained no or only a very low density of fibres. Additional areas not illustrated here are described in the text.

seen in the cuneate and external cuneate nuclei and in the lateral reticular nucleus. The raphe nuclei of the medulla contained sparse but strongly stained varicose fibres that had a similar density to the TH- and enkephalin-immunoreactive fibres.

It can be concluded that in the medulla and spinal cord, CHR-immunoreactivity coincided with TH- or enkephalin-immunoreactivity only in some areas.

4. DISCUSSION

Previous studies showed that chromogranin A-related proteins were present in endocrine cells[5,15,16,64,66,67,80], peripheral noradrenergic neurons[4,89] and (recently) in the brain using radio-immunoassay[65]. Biochemical studies have shown that these proteins were released from the first two classes of tissue[5,89].

The present study has concentrated on the central nervous system, and the results can be summarized in two general statements. First, the chromogranin-immunoreactivity was due to a family of peptides which carry common antigenic determinants. Second, the chromogranin-immunoreactivity was found throughout the nervous system, in both catecholaminergic and other neurons. The surprising abundance of chromogranin-immunoreactivity in the brain raises 3 issues. 1. The relationship between the different immunoreactive molecular species, and whether the same relationship is found in both the central nervous system and in the periphery. 2. The neurochemical characteristics of the chromogranin-immunoreactive neurons. 3. The formulation of an hypothesis for the role of chromogranin A taking account of its distribution and localization.

4.1. *Molecular nature of the CHR-antigen*

The identification of antigens localized by antisera is complicated when families of differently sized protein molecules carry the same antigenic determinant or epitope. The characteristics of the molecules recognized by the chromogranin antisera used in these experiments will be discussed in relation to the immunogen derived from the adrenal medulla. The nature of the molecules carrying these epitopes in adrenal and brain will then be compared.

4.1.1. *Characteristics of the antisera*

Immunization with highly purified chromogranin A produced antisera which recognized a number of differently sized proteins. This may have been because the proteins carry common epitopes and thus form a family, or to the presence of impurities in the immunogen.

The recognition of multiple peptides was probably not due to the presence of non-chromogranin proteins in the immunogen for the following reasons. First, the organelle-containing chromogranin A was isolated using a method which yields highly purified chromaffin granules[86]. The chromogranin A was then isolated by electrophoresis or by a procedure involving a number of extra steps to minimize the likelihood of contamination. The combination of these procedures resulted in a highly purified preparation of chromogranin A with the elimination of all polypeptides smaller than chromogranin A itself from the immunogen.

4.1.2. *Characteristics of the CHR-immunoreactive proteins in the adrenal medulla*

The antisera recognized chromogranin A together with smaller peptides not present in the immunogen. It is suggested that recognition of these smaller peptides does not represent antibodies generated in response to impurities in the immunogen for a number of reasons. For example, the pattern of staining on immunoblots was identical when lysates from either the purified chromaffin granules or unfractionated homogenates of the adrenal medulla were examined. This observation virtually eliminates the possibility that the antisera recognize impurities in the immunogen from any other type of organelle; if the antisera recognized an impurity which was not a component of the chromaffin granule then blots of the homogenate containing higher relative concentrations of the other organelle should stain differently from the granule lysate. The similarity in the staining therefore strongly suggests that all the immunoreactive polypeptides are constituents of the chromaffin granule and that they are likely to be derived from chromogranin A.

The existence of several peptides derived from chromogranin A and with common epitopes is also suggested by the work of Hortnagl et al.[43]. They found that antisera produced by immunization with either

chromogranin A or a smaller protein extracted from polyacrylamide gels, recognized 90% of the proteinaceous material in the chromaffin granule lysate, as well as the immunogens. Kilpatrick et al.[46] have found that an antiserum raised against chromogranin A also recognized smaller peptides when adrenal medullary proteins were examined.

The presence of a common epitope suggests that the peptides are likely to be derived from the same parent molecule. This suggestion is supported by two other observations. (1) The overall amino acid composition of the total proteins in the lysate does not differ significantly from the composition of purified chromogranin A (see ref. 116). Since chromogranin A accounts for only 40–50% of the proteins in granule lysates, this result suggests that the bulk of the remaining proteins have similar compositions. (2) All the polypeptides recognized by the antisera have similar isoelectric points confirming the similarity of their composition. It is probable that peptides with such similar compositions share a common origin.

The most obvious way for chromogranin A to be reduced in size in vivo is by limited proteolytic digestion, and there is evidence that such enzymes exist in the chromaffin granules[29,32,60,105]. The enzymes, which have been generally studied with respect to their ability to cleave the precursors of the enkephalins, have been variously described as having trypsin-like[24,55], atypical trypsin-like[29,60], or carboxypeptidase B-like[32] activity. Such enzymes could degrade chromogranin A into smaller fragments if it had the appropriate amino acid sequences.

Studies using an in vitro translation system also suggest that the small peptides are derived from chromogranin A by post-translational processing[46] (see also Falkensammer, G., Fischer-Colbrie, R. and Winkler, H., unpublished observations). When messenger-RNA extracted from the adrenal medulla was translated, the only product which reacted with the anti-chromogranin antiserum was a large protein similar to chromogranin A, and not the smaller products. Thus, the smaller peptides are probably not products of different genes, but derived from chromogranin A.

The chromaffin granule is known to contain 3 major species of proteins: DBH, the enkephalin precursors and the chromogranin family of polypeptides. While it has been clearly demonstrated that DBH is immunologically distinct from CHR and does not carry the epitope recognized by our antisera, the relationship between the CHR family and the enkephalin-containing proteins has not been established. It is unlikely, however, that they are biochemically related even though enkephalin and CHR are localized in the same organelle in the adrenal medulla and they share a strikingly similar distribution in parts of the brain (see below).

The largest proteins containing the enkephalin sequence have been estimated to be 50,000 dalton[53], or 34,000 dalton[77] while the size of the precursor derived from the genomic sequence is 29,000 dalton[63].

Because of the position of start and stop codons in the gene sequence, these peptides cannot be part of chromogranin A. This view is reinforced by the now known amino acid composition of preproenkephalin which differs substantially from that of chromogranin A[63]. The differences are highlighted by the recent characterization of a fragment of preproenkephalin, called synenkephalin[57], that has a pI of 7.6 as distinct from values in the range of 4.8–5.2 for the chromogranins.

In summary, the evidence supports the hypothesis that chromogranin A represents the largest of a family of related polypeptides all sharing common epitopes. The smaller molecules are probably derived from the parent by limited proteolysis, and they have the same pI and a similar amino acid composition. None of this family would appear to be related to any of the other identified protein constituents of the granules. The immunohistochemical experiments described here are therefore believed to reveal the distribution of this family of polypeptides unambiguously, but the results do not discriminate between the differently sized immunoreactive molecules.

4.1.3. *Characteristics of the CHR-antigens in brain and pituitary*

The properties of the molecules carrying the chromogranin-derived epitope were investigated in two ways. First, tissue extracts from brain and pituitary were studied by immunoblotting. Second, the identity of the epitope in the hippocampus and the adrenal was confirmed by cross-absorption.

On two-dimensional immunoblots, the immunoreactive material in bovine pituitary was indistinguishable on the basis of size and isoelectric point from that

in bovine chromaffin granule lysates. Since plasma can be excluded as the source of CHR[64], this result confirms the presence of CHR in the pituitary and shows that its synthesis and subsequent processing follows the same pathway as the one in the adrenal.

Previous studies with pituitary extracts have shown that CHR-immunoreactivity was due to the presence of a molecule that was smaller than chromogranin A[64]. Our finding of multiple peptides with the chromogranin A epitope in the pituitary could explain the broad elution profile of pituitary chromogranin when separated on the basis of size[64].

In bovine hippocampus two immunoreactive proteins were detected which were shown to be identical to the major proteins in the chromaffin granule lysate by co-migration in mixing experiments. Smaller polypeptides were not detected on two-dimensional gels, possibly reflecting the lower amounts of immunoreactive material in the tissue. In extracts from sheep brain, the immunoreactive material was similar to that in both sheep and bovine adrenals.

The identity of the antigen in brain with those in chromaffin granule lysates was confirmed by absorption experiments. The fact that extracts of the hippocampus abolished hippocampal staining in parallel with adrenal staining, and that the lysate abolished staining in the hippocampus and the adrenal in parallel, confirms that the antigens in both tissues carry the same epitopes. It also excludes the possibility that the staining is due to the presence of a contaminating antibody.

4.2. *Neuronal pathways containing CHR-immunoreactivity*

Although CHR-immunoreactivity was found in cell bodies throughout the brain, there were indications that some neurons that contained CHR-immunoreactivity in their cell bodies did not have detectable levels in their terminals (see 4.2.2.2. and 4.2.2.6.). There were also suggestions that some neurons with strongly reacting terminals had unstained perikarya (see 4.2.2.3.). Since it was not possible for ethical reasons to treat sheep with colchicine to increase the level of antigen in perikarya, the results may not represent the complete distribution of CHR-immunoreactive cell bodies.

In the following description, CHR-immunoreactive neuronal populations will be correlated with neurochemical or morphological classes defined here and elsewhere. While we have provided immunohistochemical evidence for the presence of CHR-immunoreactive material in central noradrenergic neurons using double-labeling techniques, more such direct studies will be needed for the other populations.

4.2.1. *Peripheral nervous system and endocrine cells*

The distribution of chromogranin immunoreactivity in the adrenal and pituitary reported here agrees well with the results of previous studies[16,64,66]. In addition, as would be expected if the antisera were directed against the chromogranin epitope, immunoreactivity was found in varicose terminals of peripheral noradrenergic neurons which are known to store and release chromogranin A or immunologically related peptides (see ref. 51).

4.2.2. *Central nervous system*

4.2.2.1. *Catecholaminergic neurons*. As would be predicted from the strong reaction of peripheral catecholaminergic neurons, central neurons known to contain noradrenaline were also immunoreactive. In both the locus coeruleus region and in the area of the A1 and C1 cell groups in the ventro-lateral medulla, the CHR-reactive perikarya were distributed similarly to the catecholamine containing neurons. In the locus coeruleus, the CHR-immunoreactive neurons were identified as noradrenergic by the presence of both TH and DBH in their perikarya. It is likely that the CHR-immunoreactive material was also present in the terminals of these neurons: the distribution of the TH- and CHR-containing varicose axons was identical in areas known to have a dense noradrenergic innervation from the locus coeruleus, such as the cerebral and cerebellar cortices and the hypothalamus[54].

Not all noradrenergic neurons were immunoreactive for CHR under conditions which produced the staining in the locus coeruleus. There was no reactivity apparent in the neuronal perikarya within the nucleus of the solitary tract or within the area postrema. Similarly, it was not possible to demonstrate CHR-immunoreactivity in the dopaminergic cell groups or in their terminals. Clearly, some aminergic cells contain no CHR, or the concentration was too low to be detected.

In conclusion, while CHR-immunoreactivity was present in some catecholaminergic neurons in the CNS, it was probably not present in all such neurons. It is of historical interest that 'chromogranins' were the first immunogens used to produce antisera which were subsequently applied to the immunohistochemical characterisation of neurons in the nervous system[33]. The distribution in the brain however, is not tightly linked to catecholamines, and is more widespread than would be expected if it were confined to these cell types.

4.2.2.2. *Neurons using acidic amino acids as transmitters*. Some of the neurons thought to use glutamate or aspartate as neurotransmitters also contained CHR-immunoreactivity. One such system, the hippocampal mossy fibre terminals[20,100,101], was amongst the most strongly reactive in the brain. Since most granule cells which give rise to the mossy fibres showed immunoreactivity in the Golgi apparatus, and since the density of the reactive mossy terminals was high, the possibility that the positive neurons comprise a separate sub-population can be excluded.

The pyramidal cells of the neocortex and hippocampus are also thought to use glutamate or aspartate as a neurotransmitter[30]. Like in the granule cells of the dentate gyrus, the Golgi apparatus of most pyramidal cells was strongly CHR-immunoreactive. In contrast with the granule cell terminals, however, the terminals of the pyramidal cells were probably not immunoreactive. This was inferred from two observations. First, cortical pyramidal cells have a very rich local axon collateral arborization[34], but this was not reflected by the distribution and density of the CHR-positive axons in the cortex and hippocampus. Second, some areas, like the thalamus and the red nucleus, which receive a rich cortical input showed no or few immunoreactive terminals.

The layer of termination of the lateral olfactory tract was also conspicuously CHR-immunoreactive. The lateral olfactory tract originates in the main olfactory bulb, largely from the Mitral cells, and terminates among other areas in the outer half of the plexiform layer in the olfactory tubercle and in the olfactory cortex[9,38,75]. The terminals of the pathway, at least in the olfactory cortex, probably contain aspartate or glutamate as neurotransmitters[17,18]. The layer of termination of the lateral olfactory tract contained a sharply delineated band of CHR-immunoreactivity, but since we have not studied the olfactory bulb, it remains to be established if the perikarya of Mitral cells were immunoreactive, although their terminals are almost certain to contain CHR.

It has also been suggested that some neurons in the deep cerebellar nuclei use acidic amino acid transmitters[62]. All such cells had strongly immunoreactive perikarya, but no immunoreactive terminals were found in the red nucleus, where these cells send projections.

In conclusion, neurons thought to use acidic amino acid transmitters frequently have CHR-immunoreactive perikarya and may or may not have immunoreactive terminals. The reactivity in the cell bodies was invariably confined to the Golgi apparatus.

In the context of acidic amino acid transmitters, it is pertinent to re-emphasize the amino acid composition of chromogranin. The chromogranins are particularly rich in glutamic acid[39,87], and the presence of CHR-immunoreactive material in the Golgi apparatus of many of the cells that use glutamate (or aspartate) as neurotransmitters may indicate a special function.

It should also be emphasized that the staining proposed as CHR-immunoreactivity was unlikely to have been due to the antisera recognizing the transmitter store of glutamate fixed in the tissue. An antiserum thought to be specific for glutamate stained the mossy fibres in rat brain very strongly[101], as did the antisera to CHR. However, other layers which stained strongly for glutamate[101] and which are known to contain and selectively accumulate high concentrations of acidic amino acids[102] did not stain with the CHR antiserum. The converse could be possible, however. The staining due to 'glutamate' could be due to the presence of high concentrations of the glutamic acid-rich chromogranins present in particular areas.

4.2.2.3. *Enkephalin pathways*. Antisera against 3 different opioid peptides were used to determine whether the co-existence of these small peptides and chromogranins, a feature of the chromaffin granules, was also common in brain. The 3 antisera showed essentially similar patterns of enkephalin immunoreactivity and the distribution in sheep agreed well with that obtained in other species[6,22,26,79,115]. However, the similar staining of the pallidal fibre network in the sheep using either Met-enkephalin and Met-enkeph-

alin-Arg^6-Phe^7 was in contrast to the different distribution in the rat[114,115].

In some areas, the distribution of enkephalin- and CHR-immunoreactivity in the neuropil was identical, suggesting that the peptides were present in the same nerve fibres and terminals. This similarity in distribution was marked in parts of the basal ganglia; namely in the nucleus caudatus, the globus pallidus, the putamen, the ventral pallidum and the nucleus accumbens.

The mosaic pattern produced by the differential density of enkephalin-immunoreactive axons and terminals in the neostriatum was different from the pattern found in other species[36]. Most of the neostriatum in the sheep stained reasonably uniformly, but some irregularly shaped areas surrounding myelinated axon bundles stained weakly. These weakly stained areas also had low AChE activity. In the cat, areas of the neostriatum stained strongly for the enkephalins, but weakly for AChE activity[36]. The CHR-immunoreactivity in nerve terminals closely followed the pattern and the density of the enkephalins. This was true even for details like the dendrites in the ventral neostriatum ensheathed in enkephalin or CHR-immunoreactive terminals. These characteristic dendrites, some of which may belong to striato-nigral neurons[7], have been shown to receive symmetrical synaptic contacts from the enkephalin-immunoreactive terminals. Most of the scattered enkephalin in the neostriatum also established symmetrical contacts[94]. The CHR-immunoreactive terminals established symmetrical contacts, and the immunoreactivity, like that of the enkephalins[74,94], was localized in the large granular vesicles.

The well-correlated localization in terminals contrast with the relationship in perikarya. Enkephalin-immunoreactivity has been found only in medium sized neurons in the neostriatum of the rat[74,115] and, as reported here, in similar neurons in the sheep. Most of these neurons are known to project to the globus pallidus and substantia nigra giving rise to the strong enkephalin-immunoreactivity in the neuropil of the pallidum[22]. Considering the strong CHR-staining in the globus pallidus and entopeduncular nucleus, it seemed likely that the CHR-immunoreactivity would be found in the medium sized projection neurons of the neostriatum. These neurons unexpectedly showed little or no immunoreactivity, but the large neurons, most of which do not project to the pallidum or nigra were strongly immunoreactive (see 4.2.2.5.).

One possible explanation for this apparent anomaly is the relative lack of cell organelles, including the Golgi apparatus, in the perikarya of the medium sized neurons in the neostriatum[91]. Since perikaryal CHR-immunoreactivity is largely restricted to the Golgi apparatus, the few stained saccules may not have been visible in the light microscope. This is supported by the finding that the few weakly stained medium sized neurons showed only short strands of immunoreactivity around the nucleus.

The results discussed above suggest the coexistence of enkephalins and CHR at the neostriatal and pallidal level of the sheep basal ganglia. However, the situation is more complex in the substantia nigra. Enkephalin-immunoreactive terminals were almost exclusively restricted to the areas containing the dopaminergic neurons, where they had a very similar distribution to the CHR-immunoreactive terminals. However, in most of the pars reticulata there was no visible enkephalin immunoreactivity even though the area stained strongly for CHR-immunoreactivity. This suggests that the two could be in different neuronal systems. The distribution of the CHR in the pars reticulata resembled that of substance P-like[21,58], glutamate decarboxylase[76]- and dynorphin- (see 4.2.2.4.) immunoreactivities reported in other species. Double-labeling experiments would be needed to establish whether any of these antigens co-exist in the same nerve terminal.

Another pathway thought to contain enkephalins is the hippocampal mossy fibre system[41,83,98,103], although it has also been suggested that the dynorphin peptides rather than enkephalin itself are the predominant peptides in this pathway[59] (see 4.2.2.4.). None of the antisera to the enkephalins used in this study reacted with the mossy fibres, although, as discussed above, these fibres stained strongly for CHR. The results of the absorption experiments described here suggest that, whichever opiate family proves to be in the mossy fibres, their terminals also contain CHR-immunoreactive material.

Enkephalin immunoreactivity has also been found in the oxytocin-producing neurons in the magnocellular nuclei of the hypothalamus[107]. The same cells also contain the N-terminal non-opiate segment of

the enkephalin precursor[107]. Since many of the magnocellular neurons were immunoreactive for CHR, these cells are another example of possible co-existence of the two groups of peptides.

Another possible site of coexistence of CHR- and enkephalin-immunoreactivity is within the Golgi cells of the cerebellum. Cerebellar Golgi cells have been shown to contain enkephalin immunoreactivity in other species[26,79,81,115], and the perikarya of many of these cells stained for CHR.

Enkephalin- and CHR-immunoreactivities were also distributed similarly in areas such as the central and medial amygdaloid nuclei, the raphe nuclei, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus and the substantia gelatinosa of both the spinal trigeminal nucleus and dorsal horn of the spinal cord. Although both the distribution and density of the axons and terminals were similar, without double labeling experiments it is impossible to establish which of the systems containing the variety of neuroactive substances found in these areas contains the CHR-immunoreactivity.

It is possible that the enkephalins were present in the same noradrenergic neurons in the locus coeruleus which were found to be CHR-immunoreactive. However, whether all the TH-containing cells of the locus coeruleus also contain enkephalins is not clear[12,45].

4.2.2.4. *Neurons containing dynorphin and neoendorphin immunoreactivity*. Dynorphins and neoendorphins are known to be present in the magnocellular neurons of the hypothalamus together with vasopressin[109–113]. Since many of these neurons and their terminals in the posterior pituitary were also immunoreactive for CHR, it is possible that the chromogranins co-exist with dynorphins and the posterior pituitary hormones.

Both the globus pallidus and the substantia nigra contain a high density of dynorphin-immunoreactive terminals[108,110,112]. Whether the dynorphins are in the same nerve terminals as the enkephalins is not known, but the distribution of the CHR-immunoreactivity in the sheep parallels the dynorphin pathways in the rat in both the pallidum and the nigra[108,111], while it differs from the enkephalins in the nigra[108] (see 4.2.2.3.). Other areas rich in dynorphin-immunoreactive terminals, such as the parabrachial nuclei and the nucleus of the solitary tract[45,111] were also rich in CHR-reactive terminals.

The hippocampal mossy fibres have also been reported to contain dynorphin immunoreactivity[45,59,112], and may represent another area where they are co-localized with CHR (see 4.2.2.3.).

The remote possibility exists that the antiserum to CHR recognizes dynorphin-like peptides. This is unlikely for two reasons: first, these studies have shown that several areas of the central nervous system which reacted strongly for CHR-immunoreactivity are not thought to contain the dynorphins[19,45,108,111] and second, the absorption experiments showed that prior incubation with dynorphin A did not affect the staining with the CHR antiserum.

4.2.2.5. *Cholinergic neurons*. Most of the large neurons in the neostriatum, the magnocellular neurons of the basal forebrain, the neurons of the medial septum and cranial and spinal motoneurons have all been shown to contain choline acetyltransferase (ChAT) by immunohistochemistry[2,44,47]. All these neurons were strongly immunoreactive for CHR, but others, also known to contain ChAT, e.g. in the dorsal motor nucleus of the vagus and in the medial habenula[44], were CHR-negative. Thus, there is an overlap with a neurochemically defined system, but the CHR-immunoreactivity only partially corresponds to the cholinergic marker.

It remains to be determined whether CHR can be demonstrated in areas rich in cholinergic terminals. However, it can be concluded already that the distribution of CHR is different from what would be predicted on the basis of ChAT data[2,44,47].

4.2.2.6. *GABAergic neurons*. The two CHR-positive groups of neurons most likely to use GABA as a neurotransmitter are the cerebellar Golgi cells[78] and the large neurons in the pars reticulata of the substantia nigra[68]. Other known GABAergic neurons such as the cerebellar Purkinje cells were negative. No definitive evidence has been obtained that GABAergic terminals were immunoreactive for CHR, although such terminals, along with terminals containing other neuroactive substances, are present in the CHR-rich termination areas of the neostriatal output pathways.

Evidence has recently been provided that the neurons of the reticular thalamic nuclei contain both glutamate decarboxylase and somatostatin immunoreactivity[69]. Most of these neurons were rich in CHR-immunoreactivity.

4.2.2.7. *Summary of immunohistochemical experiments*. Immunoreactive CHR has been localized selectively to certain neuronal populations and systems in the brain. Its distribution has been found to overlap partially with the distribution of many established neuroactive substances and transmitters.

The selective localization of CHR suggests that it is not a protein necessary for the storage of transmitters or hormones, and there is nothing obvious that the various cell types containing CHR have in common. The distribution of CHR thus suggests that it has a widespread role.

4.3. *Possible roles of the chromogranins*

The fact that chromogranin A was localized in the chromaffin granules proved useful in studies of hormone secretion from the gland, because its concomitant release was used to support the argument that release from the medulla occurred by exocytosis[3,14,88]. The release of the protein was not thought to have physiological significance, however, because early suggestions for the role of the chromogranins, especially chromogranin A, were related to catecholamine storage in the chromaffin granules (see ref. 88) and not for the provision of a biologically active molecule acting outside the adrenal medulla. However, it has not been possible to show unequivocally that the protein was an essential component of the storage complex, and this suggestion has been essentially abandoned.

The evidence reported here further weakens the case for an intracellular role. It may have been possible to argue that the proteins had a structural role in certain vesicles since they were discovered first in the dense-cored chromaffin granules[5], then in the noradrenergic dense-cored vesicles[23,51,85] and more recently in the hormone-storing organelles in the parathyroid gland[15] and other endocrine tissues[64,66,67].

There is, however, at least one neuronal system containing large dense-cored vesicles which have not stained for CHR-immunoreactivity: the hippocampal and cortical pyramidal cells and their axon initial segments are surrounded by GABAergic nerve terminals containing many dense-cored vesicles[93,95]. These terminals have not reacted for CHR, although they contain other peptides such as cholecystokinin and somatostatin[61,72,73,96]. Furthermore, the distribution of dense-cored vesicles in the brain is much more widespread than the CHR-immunoreactivity. The chromogranins are therefore not common to all hormone or peptide secreting cells which store their secretion products in such vesicles.

While it is still possible that members of the chromogranin family may prove to have a significant intracellular role, the indications are that its major function will be related to its release from cells. There is ample evidence that the chromogranins are amongst the proteins released from the adrenal medulla[3,5,88] and from sympathetic noradrenergic neurons[51,85,89]. The results reported here provide evidence that most neurons which react for CHR contain the proteins in their Golgi apparatus and in vesicles. The protein is therefore passaged through the secretory pathway, so its release from all the cells would be predicted. We have also provided electron microscopic evidence that CHR-containing vesicles undergo exocytosis (Fig. 5G). This suggests that the protein, or possibly smaller peptides derived from it, could play a role in synaptic transmission as well as having a role in endocrine systems.

4.3.1. *Chromogranin as a possible peptide precursor*

Neuroactive peptides are synthesized as part of larger protein molecules which are then systematically cleaved to yield both the bioactive peptide and presumed biologically inactive fragments of the parent molecule[29,32,56]. In some cases, like the enkephalins, the processing of the precursor molecule results in little free enkephalin but many enkephalin-containing peptides. So much of the enkephalin is found within other peptides, that the question of what carries the opiate activity physiologically has been raised: is it the pentapeptide enkephalin or sometimes the larger fragments of the precursor molecules containing enkephalin sequences[13,106]?

In some respects the chromogranin and the enkephalin families are similar. The largest enkephalin precursor contains several enkephalin sequences (see ref. 106). Chromogranin A would also appear to contain a number of repeating sequences since all the smaller peptides, while substantially different in size, have a narrow range of isoelectric points and are thus likely to have a similar composition. The chromogranins may be processed sequentially into smaller peptides, presumably by the action of peptidases. Like

the enkephalins, however, the processing appears to be incomplete because a full size range of immunoreactive peptides was invariably obtained in extracts of the chromaffin granules. It is not known whether the smallest immunoreactive peptide is the final product, or whether, as in the case of the enkephalins, antibodies to this product will need to be made before it will be detected. Regardless, the two parent proteins yield smaller peptides with a wide range of molecular sizes.

The enkephalin precursor can be broken down by the action of trypsin and carboxypeptidase B[106] to yield free enkephalins and other peptides. Likewise, there is evidence that the chromogranins are susceptible to proteolytic digestion[40]. It has recently been demonstrated that two related proteins, chromogranins A and B, yield similar peptides when treated with trypsin implying a common origin of the parent molecule[65]. We have preliminary evidence that chromogranin A is readily degraded by acetylcholinesterase (a proposed constituent of chromaffin granules[35]) as well as by trypsin (Ismael, Z., Chubb, I.W. unpublished observations). The enzymes responsible for the degradation of the chromogranins in vivo have not been identified. There are reports that the chromaffin granules contain peptidases which could, in principle, hydrolyze CHR but their activity appears to be low[29]. Preliminary experiments suggests that there is very little in vitro processing of the chromogranins unless exogenous enzymes are added (Ismael, Z. and Chubb, I.W. unpublished observations).

The chromogranins, like the opioid precursors[90,117], appear to be processed differently in some cells. As described above, some immunoreactive cell bodies do not appear to give rise to immunoreactive nerve terminals whereas others have terminals that are strongly positive. The simplest explanation is that the products derived from the chromogranins are different. This might also explain the puzzling results of O'Connor and Frigon[65], who have shown that the bulk of immunoassayable chromogranin A is not in synaptosomal fractions from brain homogenates, but in the cytosol fraction. Such a location might be accounted for in part by material originally in the Golgi apparatus. The full extent of peptide processing within organelles is not completely known, nor are the factors, enzymes and final products derived by the degradative processes. For example, recent evidence suggests that smaller peptides derived physiologically from such well-characterized bioactive peptides as β-endorphin[71] and vasopressin[10] might also have biological activities which are of a type totally different from that of their parent molecules. Thus, large precursors may eventually be processed right down to bioactive di-peptides.

On the basis of these examples, it is reasonable to suggest that the abundance of chromogranin immunoreactive terminals and perikarya in the nervous system, and immunoreactive cells in the pituitary and adrenal, is typical of a bioactive-peptide family with diverse functions. Although the role of the chromogranins is still unknown, their widespread but highly selective distribution opens up new possibilities for experiments to establish their function.

5. SUMMARY

Chromogranin A, the major soluble protein of the chromaffin granules, was isolated from bovine adrenals and used for immunization of rabbits. Chromogranin (CHR) immunoreactivity was studied by immunochemical and immunohistochemical methods in the adrenal, pituitary, brain and spinal cord of cattle, sheep, rats and guinea pigs using two antisera neither of which cross-reacted with dopamine β-hydroxylase. Detailed studies were done using tissues from sheep only because very weak immunoreaction was obtained in tissues from the latter two species.

Immunoblots of soluble proteins separated by two-dimensional polyacrylamide gel electrophoresis showed that the sera recognized a family of polypeptides in the adrenal which differed in size, but had almost identical isoelectric points. The patterns of immunoreactive proteins in extracts from the adrenal and pituitary were similar. Only two bands corresponding to the major high molecular weight bands in adrenal could be detected in the hippocampus which appeared to have a lower concentration of antigen. Other brain areas also showed two major immunoreactive proteins, one with a molecular weight similar to chromogranin A, and one smaller.

Adrenal chromaffin cells, peripheral noradrenergic nerve axons and terminals in the pineal gland, a proportion of the anterior pituitary cells and the neurosecretory terminals of the posterior pituitary were strongly immunoreactive. In addition, CHR-immu-

noreactivity was widely distributed in the brain and spinal cord. The reactivity was readily visible in some nerve cell bodies and in well-defined pathways and terminal fibre networks. There were neurons whose perikarya were intensely stained but whose terminal projections appeared to be negative, while in other cases, the terminals appeared rich in CHR, while the perikarya were barely stained. All chromogranin immunoreactivity was abolished by absorption of the sera with a lysate from the chromaffin granules, but was not affected by absorption with Met- or Leu-enkephalin, dynorphin$_{1-17}$, Met-enkephalin-Arg^6-Phe^7 or BAM-22P.

Electron microscopic experiments revealed that the CHR-reaction in cell bodies was almost exclusively confined to the Golgi apparatus, while in synaptic boutons it was found in large dense-cored vesicles common to many types of terminals. In the hippocampal mossy fibre terminals, the immunoreactive granulated vesicles sometimes appeared to have fused with the plasma membrane of the boutons suggesting that the CHR was being secreted by exocytosis.

The CHR-immunoreactivity was found to overlap partially with the distribution of many other neuroactive substances. The distribution of CHR was compared with that of Met- and Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7, tyrosine hydroxylase and dopamine β-hydroxylase in serial sections of the brain and spinal cord. Its distribution was also compared with that of other neurotransmitters and neuroactive peptides as described in the literature for other species. CHR-immunoreactivity was found in some of the cells thought to contain one of the following: enkephalins, dynorphins, acetylcholine, GABA, glutamate, aspartate, oxytocin and vasopressin. However, the CHR was not found in all the cells thought to contain each of these substances. For example, the very strong CHR-immunoreactivity paralleled enkephalin immunoreactivity in the globus pallidus, the central amygdaloid nucleus, the nucleus of the solitary tract and in the substantia gelatinosa of the dorsal spinal cord, but it was different in the substantia nigra and the hippocampus. Among the catecholamine cell groups, the neurons of the locus coeruleus and their presumed axons and terminals in the neocortex, hypothalamus and cerebellum were strongly CHR-immunoreactive, but no such reactivity was found in the A2 and C2 catecholaminergic neurons. A detailed analysis of the distribution of CHR thus suggests that it is present in neurons using many different transmitters.

The possible roles of CHR are discussed. The selective distribution of CHR indicates that it has a widespread but special function. It is suggested that the CHRs are a family of peptides which, like the enkephalins, enkephalin-containing peptides and other neuroactive peptides, are derived from a large precursor by limited proteolysis. The release of members of this family from endocrine cells and neurons could serve to influence target cell activity.

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