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## Corticotropin-releasing factor-like immunoreactive nerve fibers in the rat superior cervical ganglion and their fine structures

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The existence of nerve fibers containing corticotropin-releasing factor (CRF)-like immunoreactivity (CRFI) in the rat superior cervical ganglion (SCG) was demonstrated by using immunocytochemistry. They were found to be extrinsic in origin, because no CRFI neurons were seen in the SCG and decentralization resulted in the disappearance of CRFI fibers in the SCG on the operated side. These findings were also confirmed by immunoelectron microscopic analysis: CRFI fibers contained a number of small clear synaptic vesicles but were devoid of large granular and agranular vesicles. These morphological characteristics are identical to those of the preganglionic fibers. The present immunoelectron microscopic analysis revealed that most of the CRFI fibers in the SCG make synaptic contact predominantly with the dendrites of the principal cells, partly with their somas and rarely with a non-CRFI terminal. Thus, the present study provides direct morphological evidence that CRF directly influences the function of the principal cells of the SCG and that CRFI fibers are preganglionic.

### INTRODUCTION

Corticotropin-releasing factor (CRF) was originally isolated from the porcine hypothalamus<sup>17</sup> and characterized as a neuropeptide composed of 41 amino acids<sup>20</sup>. Subsequent immunocytochemical analysis has shown a wide but uneven distribution of structures with CRF-like immunoreactivity (CRFI) in the central nervous system, suggesting that this peptide is involved in a variety of functions<sup>14,15,19</sup>. In fact, central administration of CRF had various effects on the endocrine and autonomic systems such as stimulating the release of adrenocorticotrophic hormone (ACTH) and  $\beta$ -endorphin<sup>16,20</sup>, causing hyperglycemia and increasing sympathetic outflow<sup>1</sup>, mean arterial pressure and heart rate<sup>4</sup>. In addition to these central effects of CRF, a peripheral effect of CRF is also reported; an i.v. injection of CRF causes hypotension<sup>7</sup>.

This finding suggests the presence of a CRF neuron system in the peripheral autonomic nervous system. In the present study, we examined the localization of CRFI structures in the rat superior cervical ganglion (SCG) to clarify this speculation.

### MATERIALS AND METHODS

#### *Light microscopic analysis*

Eighteen male Wistar rats (about 100 g b. wt.) were divided into two groups; one was used for the analysis of the normal distribution of CRFI structures (8 rats) and the other for a fiber connection study (10 rats). Two of the rats in the first group received an i.p. injection of 10–15  $\mu$ l of colchicine (3.5 mg dissolved in 1.0 ml of saline) 24–48 h before they were killed. In the second group of rats, a unilateral transection (right side) of the cervical sympathetic trunk

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just beneath the SCG was done under pentobarbital anesthesia (40 mg/kg). The animals were kept alive for 5–7 days after the operation and were then subjected to immunocytochemistry for demonstrating CRFI structures. In addition, several sections from normal and colchicine-treated rats were subjected to immunocytochemistry for neuropeptide Y (NPY) or Leu-enkephalin (ENK), to control the effect of colchicine.

All the animals were perfused via the ascending aorta with 50 ml of ice-cold saline followed by 300 ml of Zamboni's solution<sup>21</sup>. The SCG was removed, immersed in the same fixative for 24 h at 4 °C, and then rinsed for 24 h in 0.1 M phosphate buffer containing 30% sucrose. Serial sections (20  $\mu$ m) were cut on a Vibratome (Oxford Instruments), washed in 0.1 M phosphate buffer (pH 7.4) and subjected to the indirect immunofluorescence method of Coons<sup>2</sup>. Sections were incubated overnight at 15 °C with CRF antiserum diluted (1:500) with phosphate-buffered saline (PBS). After a buffer rinse, they were incubated overnight at 15 °C with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Miles) diluted (1:1000) with PBS at 15 °C. After the buffer rinsing, the sections were mounted in a PBS-glycerine (1:1) mixture.

#### *Immunoelectron microscopic analysis<sup>18</sup>*

Four animals each weighing about 100 g were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused through the heart with saline (100 ml), followed by approximately 500 ml of a picric acid (0.2%)–paraformaldehyde (4%)–glutaraldehyde (0.05%) fixative at 15 °C. After perfusion, the SCG was then washed with several changes of 0.1 M phosphate buffer (pH 7.4), followed by 10% and 20% sucrose dissolved in the same buffer for over 1 h each time. The SCG was then frozen in liquid nitrogen and thawed in 0.1 M phosphate buffer (pH 7.4) at 15 °C. Sections (40  $\mu$ m) were cut on a Vibratome and washed in 0.1 M phosphate buffer (pH 7.4).

The sections were incubated in the following order at 4 °C unless otherwise stated: 1 h with 20% normal goat serum at 15 °C, 3  $\times$  30 min rinsing, overnight in CRF antiserum diluted to 1:500, 3  $\times$  30 min rinsing, 3 h in goat anti-rabbit IgG (Hochst) diluted to 1:50, 3  $\times$  30 min rinsing, 3 h in rabbit peroxidase–anti-peroxidase complex (PAP; Dako) diluted to 1:50 at 15 °C,

rinsing. PBS was used for all the rinsing and antibody dilutions. Normal goat serum (1% was included in the primary and second antisera, and each rinsing solution before PAP incubation. To prevent non-specific staining, 0.25% carageenan (lamb type IV, Sigma) was also used for the second and third rinsing each time before PAP incubation.

The sections were incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Dotite, Japan) dissolved in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01% hydrogen peroxide for 3–6 min at 15 °C following preincubation with the same solution omitting hydrogen peroxide for 1 h in a dark room at 4 °C. After washing, they were postfixed for 1 h in 1% OsO<sub>4</sub> in 0.1 M, pH 7.4 phosphate buffer and then dehydrated. The sections were stained with 1% uranyl acetate at the 70% alcohol dehydration stage. The tissue was flat-embedded on siliconized slides in Epon 812. After light microscopic observation, ultrathin sections were made serially, and mounted on Formvar-coated single slot (2  $\times$  1 mm) grids and stained with lead.

#### *Specificity of the antiserum*

CRF antiserum was produced in rabbits injected with synthesized CRF conjugated to porcine thyroglobulin. The specificity of the antiserum was tested using a radioimmunoassay, which confirmed that this antiserum reacted well with rat CRF but did not cross-react with ovine CRF, sauvagine and urotensin I, or with other peptides including somatostatin (SOM), substance P (SP), neurotensin (NT), renin, glucagon, cholecystokinin-8 (CCK), luteinizing hormone-releasing hormone and ACTH (T. Shibasaki, unpublished data). Specificity of the antiserum was also examined histochemically by an adsorption test as follows: (1) replacement of the specific antiserum with normal rabbit serum, (2) omission of the specific serum, and (3) adsorption of anti-CRF serum to CRF, ENK, SOM SP, NT or CCK. Adsorption was carried out at 4 °C at the final dilution, with each peptide ( $10^{-5}$  M), followed by centrifugation at 10,000 g for 30 min. The supernatant was used for the adsorption test. Structures that satisfied all of the following criteria were taken to be positive for CRF: no immunostaining was seen when the specific serum was replaced by normal serum, when the specific serum was omitted and when the specific serum was adsorbed

with CRF, but no decrease was seen when the sections were incubated with antiserum against CRF adsorbed by any of the other peptides tested.

## RESULTS

### *Distribution of CRFI structures in the SCG*

A plexus of CRFI fibers was detected in the SCG (Fig. 1A). These fibers had a varicose appearance and were unevenly distributed in the SCG. Areas innervated by CRF fibers were intermingled with those poorly innervated (Fig. 1A). Most of the immunoreactive fibers surrounded the somas of the principal cells, while no CRFI fibers were detected near the so-called small intensely fluorescent cells. CRFI fibers were also seen in the capsule of the SCG (Fig. 1B). They were thicker than those seen within the SCG and had a smooth appearance. No immunoreactive cells were seen in the SCG even in the animals treated with colchicine. On the other hand, NPY- or ENK-labeled cells were seen in great number in the SCG and in the colchicine-treated animals; an enhanced immunoreaction for them was identified.

### *Fiber connection study of CRFI fibers in the SCG*

The distribution of the above two types of CRFI fiber together with the lack of immunoreactive cells in the SCG suggested that they are extrinsic in origin. This was supported by the findings that decentralization of the SCG resulted in the disappearance of both types of CRFI fiber on the operated side (Fig. 1C). Thus most of the varicose type of CRFI fiber are probably terminal fibers, while those located in the capsule of the SCG are passing fibers that enter the SCG.

### *Fine structure of CRFI fibers*

CRFI fibers were readily identified under the electron microscope even at a low magnification because of strong electron dense precipitations. They were found exclusively in neuronal elements. Immunoreactive end products were found diffusely throughout the cytoplasm, and were mainly associated with the surface membrane of the cellular organelles, such as mitochondria and small lucent synaptic vesicles.

CRFI fibers were about 0.5–2.0  $\mu\text{m}$  in diameter and were exclusively filled with small electron lucent vesicles. Many of these fibers were ensheathed by a

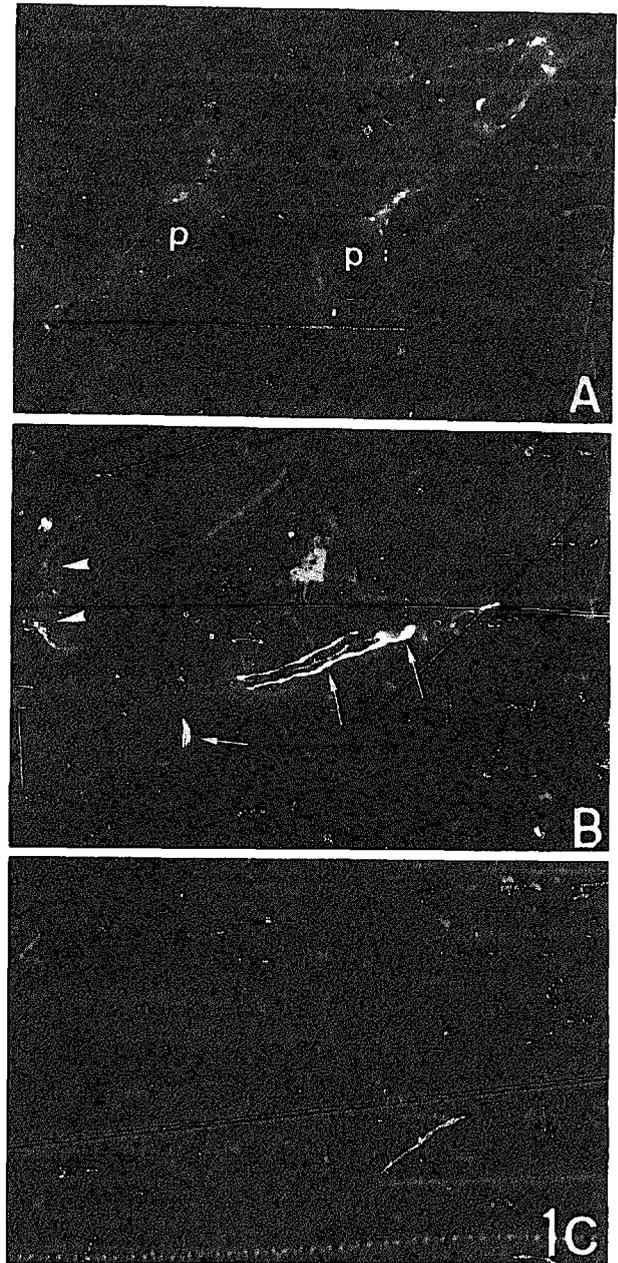


Fig. 1. A: fluorescence photomicrograph showing CRFI varicose fibers that run among and surround the principal cells (P) of the SCG. B: fluorescence photomicrograph showing CRFI nerve fibers (arrows) in the capsule of the SCG. Note their smooth appearance. Arrowheads show CRFI varicose fiber plexus in the SCG. C: fluorescence photomicrograph showing the disappearance of CRFI structures after decentralization of the SCG. A,  $\times 175$ ; B,  $\times 85$ ; C,  $\times 70$ .

glial process and in some instances, they lost their ensheathment and formed synaptic contact with dendrites (Fig. 2A) or directly with the somas (Fig. 2B). Dendrites with which CRFI fibers formed a synapse were large in size (about 1–2  $\mu\text{m}$ ) and rich in cyto-

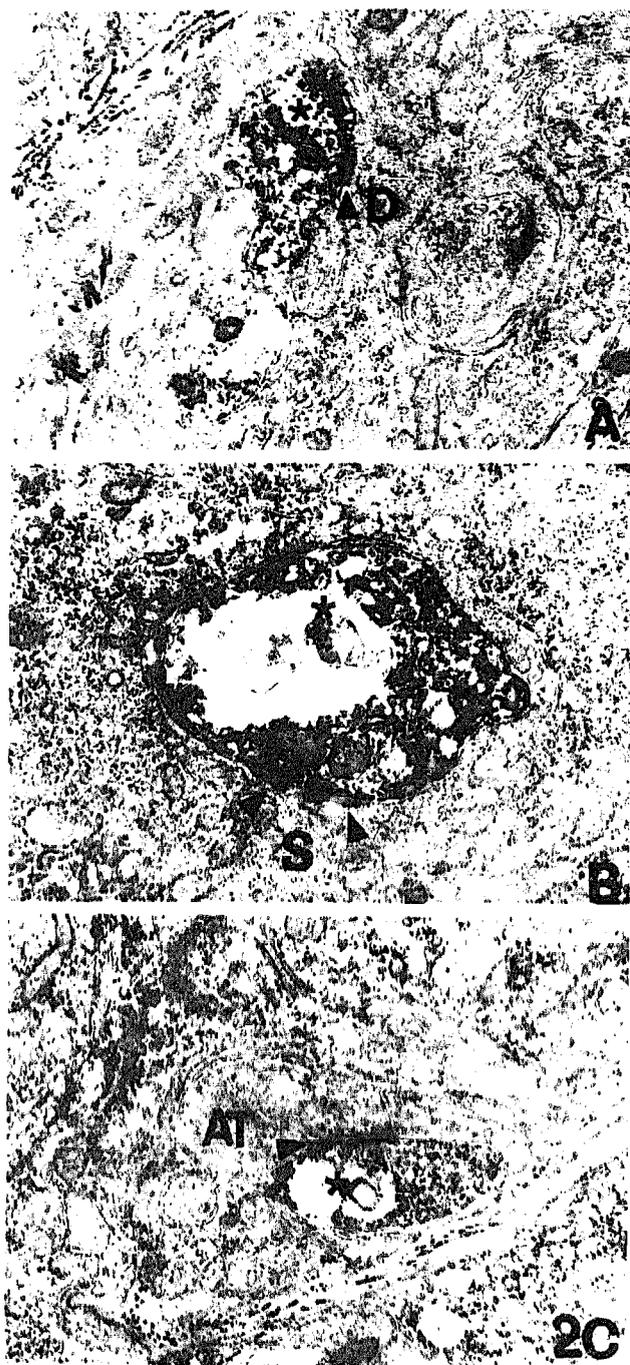


Fig. 2. Electron micrographs showing axodendritic (A), axosomatic (B) and axoaxonic (C) synapses (arrowheads) between CRFI fibers (asterisks) and neuronal elements of the SCG. AT, non-CRFI terminal; D, dendrite of the principal cell; S, soma of the principal cell. A,  $\times 16,900$ ; B,  $\times 20,000$ ; C,  $\times 16,900$ .

plasmic organelles (Fig. 2A), suggesting that they belong to the proximal segment. Up to 59 CRFI terminals that formed synaptic contact with the neuronal elements were counted. Among them about 77% were axodendritic and about 20% were axosomatic. An axoaxonic-like contact was rarely identified (3%)

(Fig. 2C). In this case, CRFI terminals seemed to be presynaptic in structure, because an aggregation of synaptic vesicles on the contact membrane was noted in the CRFI terminal. All the synapses formed by the CRFI fibers were symmetrical. Pre- and postsynaptic thickening was not conspicuous, though enlargement of the synaptic cleft and intersynaptic filaments was clearly observed. Dendrites or somas in contact with CRFI fibers had an electron lucent cytoplasm (Fig. 2A,B). Organelles such as the mitochondria, ribosomes, granular endoplasmic reticulum and Golgi apparatus were dispersed throughout the electron lucent matrix (Fig. 2A,B). Since they usually lacked the large granular or agranular vesicles, they belonged to the principal cell somas and their dendrites. On the other hand, axon terminals with which CRFI terminals formed close contact were about  $0.5\text{--}1\ \mu\text{m}$  in diameter and were filled with small electron lucent vesicles (Fig. 2C).

#### DISCUSSION

The present study demonstrated the presence of CRFI fibers in the SCG; they are extrinsic in origin, because decentralization of the SCG resulted in their disappearance and no CRFI neurons were detected even in the colchicine-treated rats. The immunoelectron microscopic analysis further supported this conclusion. CRFI fibers were filled with small clear synaptic vesicles but were devoid of large granular and agranular vesicles. These ultrastructural characteristics are very similar to those of the preganglionic fibers<sup>3</sup>.

The presence of CRFI neurons in the intermediolateral nucleus of the thoracic cord has been reported by several authors<sup>8,13,14</sup>. Thus, it is likely that the origin of CRFI fibers in the SCG lies here.

CRF has been suggested to be involved in the regulation of autonomic functions. For example, an i.v. injection of CRF causes hypotension. On the other hand, noradrenaline located in the principal cells causes vasoconstriction. Therefore it is probable that CRFI-containing preganglionic fibers inhibit the function of the principal cells. Our immunoelectron microscopic study gave ultrastructural support for this hypothesis, because most of the CRFI fibers in the SCG made synaptic contact with the principal cells of the SCG, indicating that CRF directly influ-

ences the principal cells. Accordingly, some of the effects of CRF on autonomic functions may be attributed to the peripheral effects via this system. However, CRF may not directly influence all principal cells of the SCG, because areas with many CRFI fibers and those with few CRFI fibers were intermingled. To elucidate the action of CRF in the SCG, it is necessary to clarify the characteristics of the principal cells with which CRFI fibers make contact.

The present study further demonstrated the presence of an axoaxonic-like contact between CRFI fibers and non-CRFI fibers, although this contact was rarely seen. Axons with which CRFI fibers made synapses may originate from the spinal cord as preganglionic fibers, because the characteristics of these fibers were very similar to those of the preganglionic fibers in that they were filled with small clear synaptic vesicles. The CRFI terminal of these fibers also seemed to be postsynaptic in structure. Accordingly, the activity of CRFI fibers originating from the spinal cord as preganglionic fibers may be inhibited presyn-

aptically by the terminals that also belong to preganglionic fibers at the level of the SCG.

Recently, evidence for the coexistence of multiple neuromodulators or neurotransmitters in the same neuron has been obtained<sup>5,6,11</sup>. The present study demonstrated that CRF is one of the components of the preganglionic fibers projecting to the SCG. Acetylcholine (ACh)<sup>10</sup> and ENK<sup>12</sup> are also present in the preganglionic fibers of the SCG. In addition, the coexistence of these two substances in single neurons of the lateral horn of the spinal cord was shown<sup>9</sup>. These findings suggest the possibility of a coexistence of CRF, ENK and ACh in the same preganglionic fiber. However, this possibility is unlikely, because the distribution pattern of CRFI in the SCG differs from those of ENK and ACh. The areas rich in CRFI and those poor in CRFI are intermingled, whereas ENK and ACh are distributed much more evenly in the SCG<sup>10,12</sup>. A further analysis is required to resolve this problem.

## REFERENCES

- 1 Brown, M.R., Fisher, L.A., Spiess, J., Rivin, C., Rivier, J. and Vale, W., Corticotropin-releasing factor: actions on the sympathetic nervous system and metabolism. *Endocrinology*, 111 (1982) 928-931.
- 2 Coons, A.H., Fluorescent antibody method. in J.F. Danielli (Ed.), *General Cytochemical Methods*, Academic, New York, 1958, pp. 399-422.
- 3 Eränkö, O., Soinila, S. and Pääväranta, H. (Eds.), *Histochemistry and Cell Biology of Autonomic Neurons, SIF Cells and Paraneurons, Advance in Biochemical Psychopharmacology*, Vol. 25, Raven, New York, 1980.
- 4 Fisher, L.A., Rivier, J., Rivier, C., Spiess, J., Vale, W. and Brown, M.R., Corticotropin-releasing factor (CRF): central effects on mean arterial pressure and heart rate in rats. *Endocrinology*, 110 (1982) 2222-2224.
- 5 Hökfelt, T., Fuxe, K. and Pernow, B. (Eds.), *Coexistence of Neuronal Messengers: a New Principle in Chemical Transmission, Progress in Brain Research*, Vol. 68, Elsevier, Amsterdam, 1986.
- 6 Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M. and Schultzberg, M., Peptidergic neurons, *Nature (London)*, 284 (1980) 515-521.
- 7 Karin, N.H., Shelton, S.E., Kraemer, G.W. and McKinney, Jr., W.T., Corticotropin-releasing factor causes hypotension in Rhesus monkeys. *Lancet*, 2 (1982) 1042.
- 8 Krukoff, T.L., Segmental distribution of corticotropin-releasing factor-like and vasoactive intestinal polypeptide-like immunoreactivities in presumptive sympathetic preganglionic neurons of the cat. *Brain Research*, 382 (1986) 153-157.
- 9 Krukoff, T.L., Ciriello, J. and Calaresu, F.R., Segmental distribution of peptide-like immunoreactivity in cell bodies of thoracolumbar sympathetic nuclei of the cat. *J. Comp. Neurol.*, 240 (1985) 90-102.
- 10 Lindh, B., Staines, W., Hökfelt, T., Terenius, L. and Salvaterra, P.M., Immunohistochemical demonstration of choline acetyltransferase immunoreactive preganglionic nerve fibers in guinea pig autonomic ganglia. *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 5316-5320.
- 11 Lundberg, J.M. and Hökfelt, T., Coexistence of peptides and classical neurotransmitters. *Trends Neurosci.*, 6 (1983) 325-333.
- 12 Matsuyama, T., Wanaka, A., Kanagawa, Y., Yoneda, S., Kimura, K., Hayakawa, T., Kamada, T. and Tohyama, M., Two discrete enkephalinergic neuron systems in the superior cervical ganglion of the guinea pig: an immunoelectron microscopic study. *Brain Research*, in press.
- 13 Merchenthaler, J., Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. *Peptides*, 5 (suppl. 1) (1984) 53-59.
- 14 Merchenthaler, J., Hynes, M.A., Vigh, S., Schally, A.V. and Petrusy, P., Immunocytochemical localization of corticotropin releasing factor (CRF) in the rat spinal cord. *Brain Research*, 275 (1983) 373-377.
- 15 Olschowka, J.A., O'Donohue, T.L., Muller, G.P. and Jacobowitz, D.M., Hypothalamic and extrahypothalamic distribution of CRF-like immunoreactive neurons in the rat brain. *Neuroendocrinology*, 35 (1982) 305-308.
- 16 Rivier, C., Brownstein, M., Spiess, J., Rivier, J. and Vale, W., In vivo corticotropin releasing factor induced secretion of adrenocorticotropin,  $\beta$ -endorphin and corticosterone. *Endocrinology*, 110 (1982) 272-278.
- 17 Schally, A.V., Arimura, A., Redding, J.W., Chihara, K.,

- Gordin, A., Huang, W.Y. and Saffran, M., Purification of corticotropin releasing factor from porcine hypothalamus, *59th Annu. Meet. Endocrinol. Soc.*, (Abstract), 1977, p. 95.
- 18 Somogyi, P. and Takagi, H., A note on the use of picric acid-paraformaldehyde-glutaraldehyde fixative for correlated light and electron microscopic immunocytochemistry, *Neuroscience*, 7 (1982) 1779-1783.
- 19 Swanson, J. W., Sawchenko, P.E., Rivier, J. and Vale, W., Organization of ovine corticotropin releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study, *Neuroendocrinology*, 36 (1983) 165-186.
- 20 Vale, W., Spiess, J., Rivier, C. and Rivier, J., Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and  $\beta$ -endorphin, *Science*, 213 (1981) 1394-1397.
- 21 Zamboni, L. and De Martino, C., Buffered picric acid formaldehyde: a new rapid fixative for electron microscopy, *J. Cell Biol.*, 35 (1967) 148A.