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Light and Electron Microscopic Localization of Substance P-Like Immunoreactivity in the Cerebral Ganglion of Locust with a Monoclonal Antibody

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Summary. The occurrence of substance P-like immunoreactivity was studied in the locust brain at light and electron microscopic level using monoclonal IgG fraction to substance P. Small immunoreactive perikarya have been found beside the medial neurosecretory cells in horizontal brain sections. Widespread immunoreactivity was also observed in the protocerebral neuropil notably in the central body and bordering on the corpora pedunculata. The reaction endproduct appeared as fine, more or less round particles in the central body, and as coarse varicosities and wavy fibres bordering the peduncles. The roundish particles probably represent nerve terminals, while the wavy fibers correspond to neural processes. In the vicinity of the β lobe immunoreactivity was not observed. Electron microscopically, a number of immunoreactive terminals were found in the protocerebral neuropil. The reaction endproduct was accumulated mostly in large dense core granules/average diameter 80 nm/however reaction endproduct was also observed on the external surface membranes of clear vesicles and mitochondria. Our results suggest the widespread occurrence of a substance-P immunoreactive neuropeptide in the cerebral ganglia of the migratory locust.

Introduction

The widespread occurrence of neuropeptides and "peptide-factors" is well known in the neuroendocrine system of insecta (Mordue and Stone 1979; Stone and Mordue 1980). However, only a few have been characterized chemically (Mordue and Stone 1981). More than twenty peptides with known sequence have been suggested to play a role as neurotransmitters or neuromodulators in mammals (Hökfelt et al. 1977) but until now only one pentapeptide, proctolin, has been identified as a putative neurotransmitter in the nervous system of insecta (Walker 1978). The scarcity of data

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on peptides in insecta is partly explained by the extremely low amount of neuropeptides in the small organs of these animals: thus the isolation, purification and analysis of the biologically active material raises difficulties. Following the synthesis of proctolin (Andersen et al. 1967) and production of a specific antiserum the immunocytochemical localization of this material in the cockroach became possible (Eckert et al. 1981). Recently it was shown that antisera produced against mammalian neuropeptides also detect immunoreactive material in invertebrate animals (Duve and Thorpe 1980). Thus somatostatin-like material was demonstrated in the neurosecretory cells of locust (Doer-Schott et al. 1978) and neurophysin and vasopressin-like material was found in the suboesophageal ganglion in the same animal (Remy et al. 1979). Very recently immunohistochemical evidence has been reported that some gastro-entero-pancreatic peptides from vertebrates are detectable in the nervous system of the larva of the hoverfly, Eristalis aeneus (El-Salhy et al. 1980). In a previous electron microscopic study a great number of putative "peptidergic" terminals were found in the brain of the locust (Benedeczky 1981) thus we began to examine the distribution of neuropeptides using antibodies directed again well characterized antigenic determinants. Here we report the distribution and fine structural localization of substance P immunoreactive material in the locust cerebral ganglion.

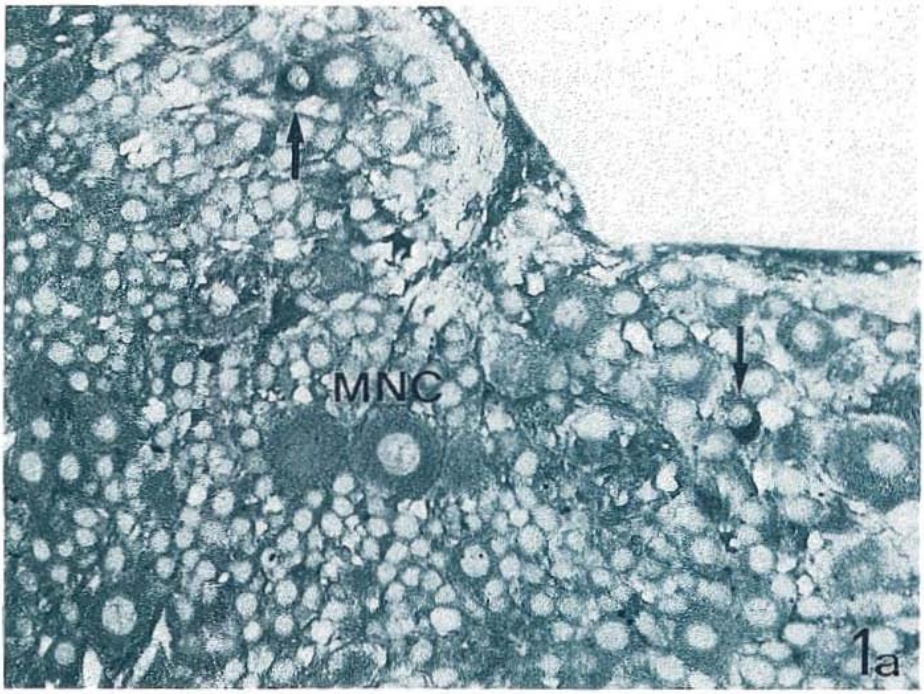
Material and Methods

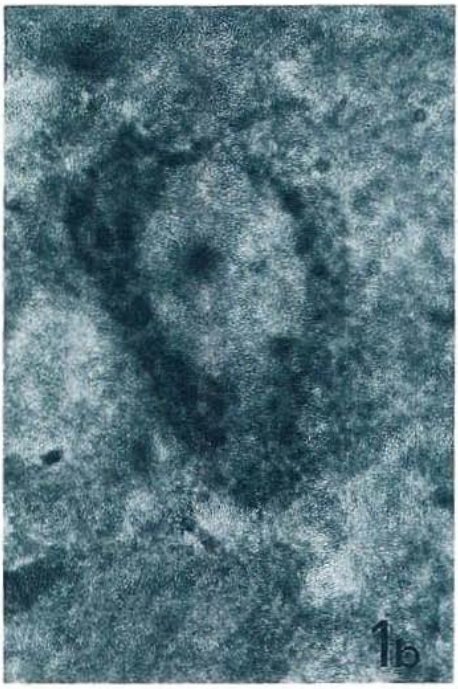
Studies were carried out on both sexes of mature Locusta migratoria (migratorioides R.F.) bred at the Institute. The animals were decapitated and the cerebral ganglion was prepared in an insect Ringer solution. The following fixatives were used; 2.5% glutaraldehyde for 4 h or 4% paraformaldehyde with 0.05% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). In a later series of experiments a buffered picric acid-paraformaldehyde-glutaraldehyde fixative for correlated light and electron microscopic immunohystochemistry (Somogyi and Takagi 1982) was used. As this later fixative gave better staining the results are illustrated with pictures from this material. For light microscopy the cerebral ganglion was dehydrated and embedded in Durcupan (Fluka) resin.

A series of semithin sections through the whole ganglion was cut and mounted on slides for postembedding immunocytochemistry. The resin was removed using ethanolic sodium hydroxide then the slides were treated with phosphate buffered saline (PBS, pH 4 and pH 7).

Immunocytochemical incubation of the slides was carried out at room temperature as follows: 30 min in 20% normal rabbit serum (Capel), 20 min wash in PBS, 90 min in monoclonal rat-mouse hybrid anti-substance P IgG fraction (clone N Cl/34 HL, diluted 1:150, Cuello et al. 1979), wash for 10 min in PBS, incubate for 60 min in rabbit anti-mouse IgG, conjugated with horseradish peroxidase (Miles) diluted 1:100, wash 30 min in PBS. All dilutions were made with PBS, which was also used for washing.

For electron microscopy small slices were cut after fixation and washed in several changes of 0.1 M phosphate buffer. Incubation for preembedding immunocytochemistry was carried out using the same sera and reagents but incubation in the anti-substance P IgG fraction was carried out overnight at 4° C. Peroxidase activity was localised as described previously (Somogyi et al. 1982). Sections were then washed 3×15 min in phosphate buffer. Semithin sections, on slides, were treated with 0.1% OsO_4 in phosphate buffer for 5 min. Tissue slices which were stained using the preembedding method were treated with 2% OsO_4 in phosphate buffer for 1 h, then washed in phosphate buffer, dehydrated and embedded in Durcupan (Fluka). To enhance contrast 1% uranyl acetate was included in the 70% ethanol for 40 min. No lead staining was used. To test the specificity of the reaction, incubations were carried out either omitting the monoclonal IgG fraction or replacing it with normal rat serum at dilutions 1:150 and 1:1,000.





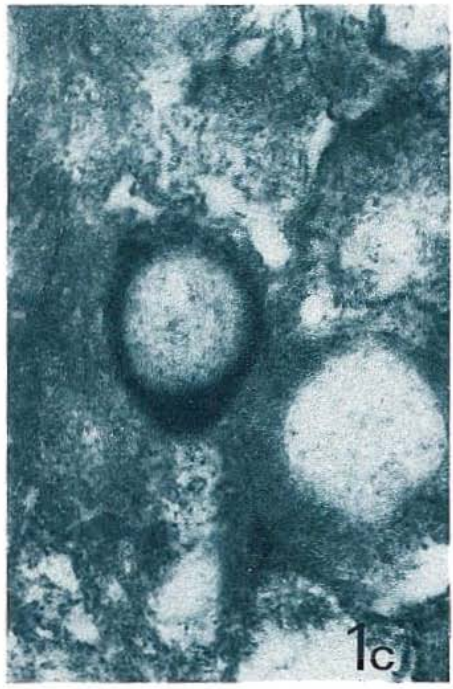


Fig. 1 a. Horizontally sectioned cerebral ganglion of the migratory locust. Substance P immunoreactive cells (→) can be seen in the vicinity of medial neurosecretory cells (MNC). × 200. **b** High magnification light micrograph of a substance P neuron containing fine granulated immunoreactive material. × 1,000. **c** Signet-ring shape immunoreactive cell from the same area of the locust brain. × 800

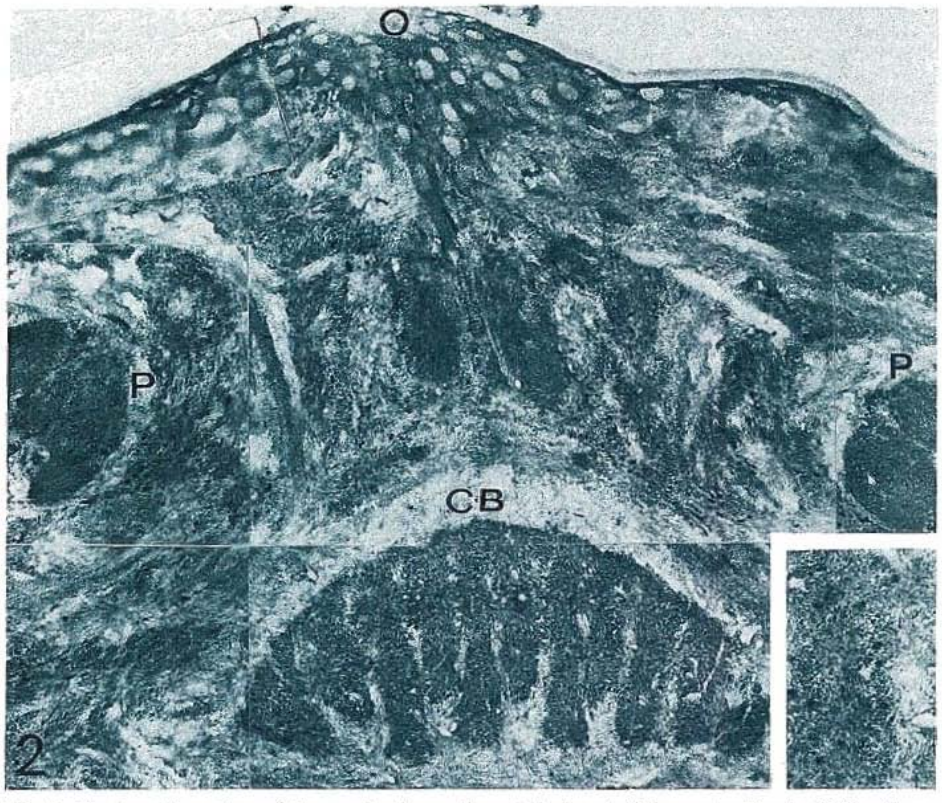
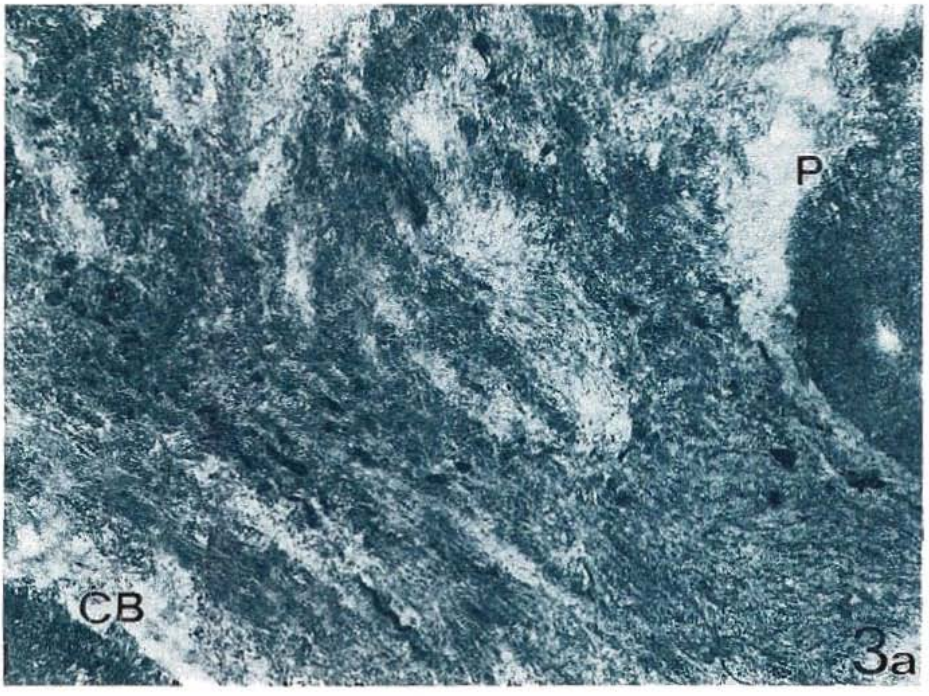


Fig. 2. Horizontal section of the cerebral ganglion at the level of the central body (*CB*). Abundant immunoreactive material is found in the central body as well as around the corpora pedunculata (*P*). *O*, ocellus. Insert: grains of reaction endproduct in the central body. × 400

Results

The monoclonal IgG fraction used in this study has been shown to recognise the C terminal portion of substance P (Cuello et al. 1979). As the same antigenic site may be present in several molecules we refer to the specific staining observed as substance P immunoreactivity. No endogenous peroxidase activity was observed in the controls.

Substance P Immunoreactivity was not detectable in the nerve cells of the surface of the horizontally sectioned brain. The substance P immunoreactive cells occur mainly in the pars intercerebralis, alongside the large neurosecretory cells (Fig. 1a). These relative small cells have oval or pear-shaped form, their diameter ranging between 15–20 μm. At high magnification (Figs. 1b and c) a fine granulated reaction product was observed in the perikarya. The most abundant reaction product was accumulated in the protocerebral neuropil at the level of the central body (Fig. 2). A fine granular evenly distributed immunoreactivity was observed in the whole cross section of the central body (Fig. 2, and Fig. 2 insert). Strong immuno-



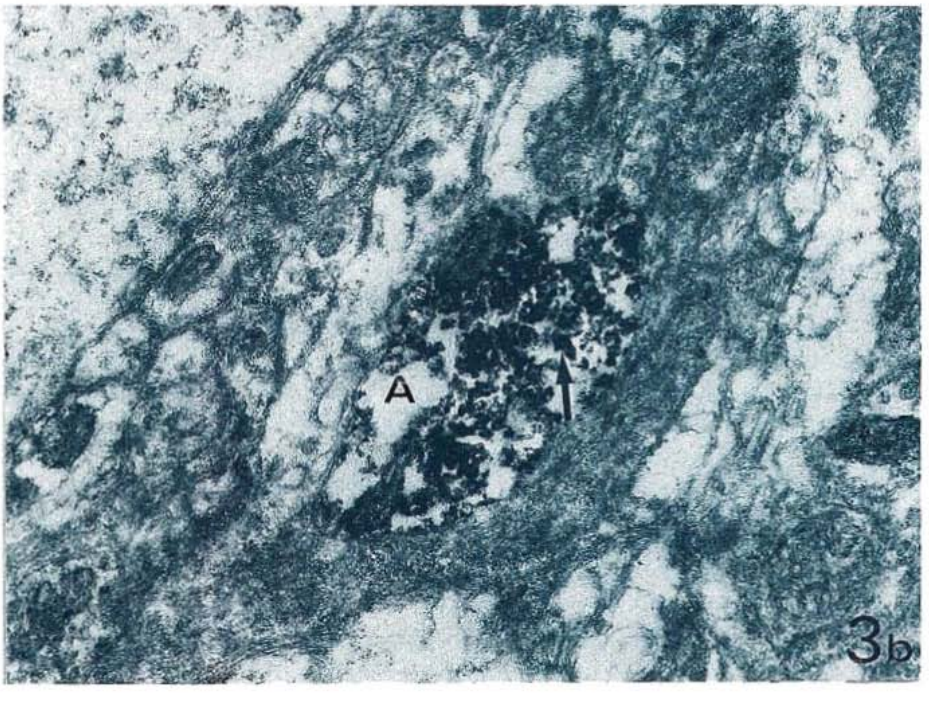


Fig. 3a. Coarse varicosities and fibres can be seen in the neuropil area between the central body (*CB*) and corpora pedunculata (*P*). × 500. **b** Ultrastructural localization of substance P immunoreactivity in the protocerebral neuropil of the locust. Densely labelled vesicles (→) are present in the axon terminal (*A*). × 40,000

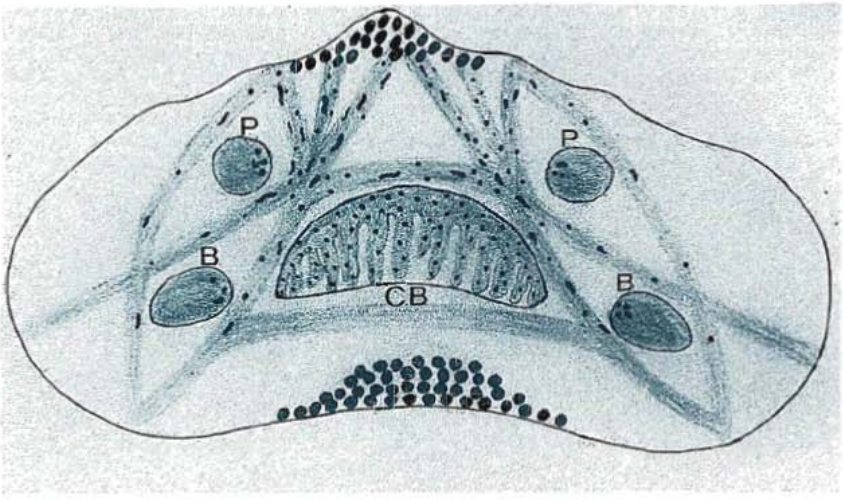


Fig. 4. Diagram of the horizontally sectioned cerebral ganglion of the locust. Dense grains and lines represent the substance P-like immunoreactivity in the plane of central body (*CB*). *P*, peduncles; *β*, beta lobe. Groups of roundish black cells at the top and bottom of the diagram are not immunoreactive

reactivity was also found in the neuropil lying between the central body and corpora pedunculata (Fig. 2). Coarse varicosities and fibers could be observed in this area (Fig. 3a) and occasionally long characteristic wavy segments of immunoreactive axons were also conspicuous (Fig. 3a). Knob-like immunoreactive particles (probably boutons) were frequently observed in this area (Fig. 3a). The peduncles themselves rarely contained immunoreactive elements. The orientation of the immunoreactive axons is mainly posterio-anterior, and some axons reach the cortex of the frontal ocellus (Figs. 2 and 4). The immunoreactivity usually disappears in the depth of the *β* lobe and only a small amount of reaction product was detectable in this area of the protocerebral neuropile. At the electron microscopic level electron dense reaction product was observable in nerve terminals of the protocerebral neuropil (Fig. 3b). The round granulated vesicles (80–100 nm in diameter) contained reaction endproduct over their core. The clear vesicles had electron dense reaction product associated with the external surface of their membrane. Other organelles (as for example mitochondria) in the terminals were also labelled with a small amount of reaction product (Fig. 3b) on their external membrane surfaces.

Discussion

Substance P is widely distributed in the central nervous system of vertebrates (Leeman and Mroz 1974; Takahashi and Otsuka 1975) and it may be a neurotransmitter in primary sensory systems (Stern 1963; Krnjevic 1974;

Leeman and Mroz 1974; Takahashi and Otsuka 1975). Little is known about the occurrence of substance P in invertebrates. Substance P-like material was demonstrated in the brain of the hoverfly larva (El Salhy et al. 1980). In addition substance P like immunoreactivity was found in the gut of the snail by Van Noorden et al. (1980). Comparing our study with previous data (El Salhy et al. 1980) the main difference is that we could demonstrate substance P-like immunoreactivity in the protocerebral neuropil while El Salhy et al. (1980) detected it only in the somata of a few nerve cells. This difference is all the more surprising since in both experiments used a similar immunocytochemical method (unlabelled antibody-enzyme and only the antisera, fixation procedure and embedding media were different. Using only glutaraldehyde fixation we observed a relatively weak immunoreactivity. Introducing the picric-acid containing fixative (Somogyi and Takagi 1982) the immunoreactivity was observed in the same regions in the brain but the reaction product was more abundant and its density, was higher. As regards the occurrence of immunoreactive nerve cells, we could find substance P-like immunoreactivity only in a few somata, result in agreement with those of El Salhy et al. (1980).

There may be more substance P containing nerve cells in the locust brain, but the concentration of the neurotransmitter may not be high enough for the immunocytochemical detection. It is also possible, that substance P is present in a masked form (bound to other molecules) in the perikarya thus the detection with an antibody recognising the C terminal part is not possible. The widespread occurrence of the substance P-like immunoreactivity in the locust brain, as for example in the central body, suggests that it may play a role as a mediator or modulator. However, in contrast to mammals, where at least two substances have been found to show substance P activity (Ben Ari et al. 1979) nothing is known about the substance responsible for the immunoreaction in invertebrates. Thus the function of a substance P immunoreactive system remains uncertain. The central body, which is the main neuropil area where substance P immunoreactivity was found is well known as a higher motor center (Bullock and Horridge 1965). On the other hand the close morphological contact of substance P-like immunoreactive fibers with the ocellus suggests that substance P, as in the case in mammals, may occur also in sensory systems. In light micrographs it is clear, that the appearance of substance P-like immunoreactivity is usually finely granulated and bulbous varicosities are present along the fibers. It is likely that these structures represent nerve terminals in the central body and also in other parts of the protocerebral neuropil. Electron microscopic examinations have confirmed this assumption since the electron dense reaction product was observed in nerve terminals. In these terminals, as in mammals (Pickel et al. 1977, 1979; Somogyi et al. 1982), the electron dense reaction product was localised mainly in granules having a diameter of 60–80 nm. Reaction product was also observed in the axoplasm and on the surface of mitochondria and clear vesicles. Since large granulated vesicles (average diameter 80 nm) are very common in the locust brain they may be responsible for the storage of chemical mediators (Benedeczky 1981)

and we suppose, that substance P immunoreactive material is also stored in these granules. The immunoreactive terminals also contained small clear vesicles containing no reaction end product. This raises the possibility that the immunoreactive material occurs together with some other substance(s) in the terminals.

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