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SHORT COMMUNICATION

PERIPHERAL NEUROSECRETORY CELLS OF INSECTS CONTAIN A NEUROPEPTIDE WITH BURSICON-LIKE ACTIVITY

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In insects, neurosecretory cells have been found not only within ganglia of the central and stomatogastric nervous system, but also in the peripheral nerves (Fifield & Finlayson, 1978; Wasserman, 1985; Baudry-Partiaoglou, 1987). These peripheral neurones and their processes along peripheral nerves contain electron-dense granules and stain with various dyes for neurosecretory cells, suggesting that they produce, store and probably release neurosecretory material. The nature of this material, however, is unknown. Only the results of Raabe (1986), showing that some abdominal peripheral neurosecretory cells of three insects are glucagon-immunoreactive, have suggested that such cells are peptidergic. We demonstrate in this study that a group of peripheral neurosecretory cells in crickets contains such a peptide. Moreover, we are able to suggest a function for this peptide and thus for an identified set of peripheral neurosecretory neurones.

We have discovered a group of three, hitherto undescribed, peripheral neurosecretory cells (PNC) in the neck region of crickets (*Gryllus bimaculatus*) with the aid of cobalt chloride backfills (Tyrer & Altman, 1974) of prothoracic and suboesophageal nerves, followed by silver intensification (Bacon & Altman, 1977) (Fig. 1). In addition, a network of fibres with superficial varicosities is revealed on all anterior nerves of the prothoracic ganglion and one nerve of the suboesophageal ganglion when PNC are labelled anterogradely (Figs 1, 2A,B). Electron micrographs of the PNC (Fig. 2C) reveal a cytoplasm containing electron-dense vesicles, mitochondria, Golgi complexes and rough-surfaced endoplasmic reticulum, indicating that these cells are in an active metabolic phase. Sections of the nerves covered with PNC projections show processes embedded in the neural sheath of the nerve, filled with electron-dense vesicles of the same type as those in the PNC cytoplasm (Fig. 2D). Many PNC fibres enter the median nerve, an unpaired nerve between the neck connectives, *via* its branches, the transverse nerves (Fig. 1). In insects, median and transverse nerves are neurohaemal organs (NHO) where neurosecretory material is stored and released into the haemolymph (Gupta, 1983).

Key words: insects, peripheral neurosecretory cells, bursicon, neuropeptides.

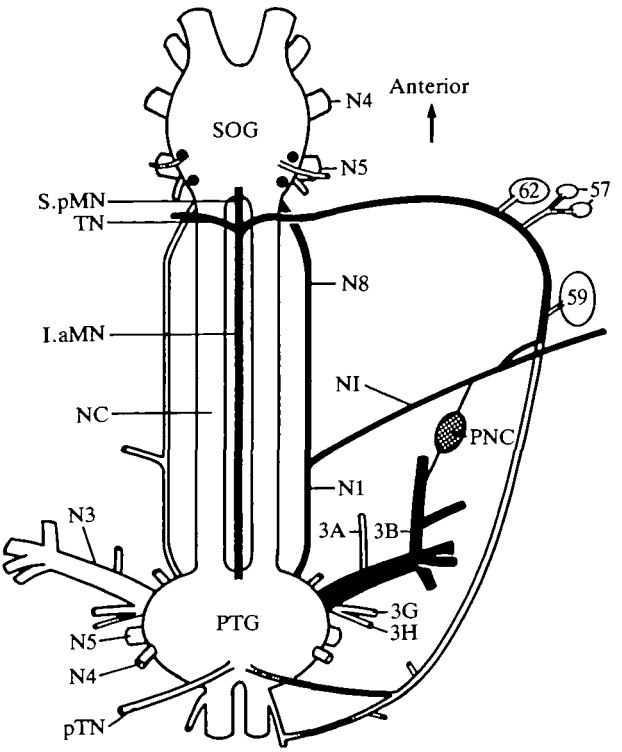

Fig. 1. Innervation scheme of cricket neck region (adapted from Honegger *et al.* 1984). The shaded structure contains the three peripheral neurosecretory cells (PNC); black nerves contain varicose fibres from the PNC and have tanning activity in the ligated fly bioassay (see text). SOG, suboesophageal ganglion; the two black dots next to and posterior to nerve N5 on either side indicate the approximate position of the somata of M62 motoneurones; PTG, prothoracic ganglion; NC, neck connectives; I.aMN, anterior median nerve; S.pMN, suboesophageal posterior median nerve; TN, transverse nerve; pTN, posterior TN; 62,57,59, neck muscles; for each ganglion, nerves are numbered from anterior to posterior as N1 to N8.

The median nerve (MN) between the suboesophageal and prothoracic ganglia of adult crickets with its two branches, the transverse nerves (TN) (Fig. 1), contains bursicon-like activity (Table 1; Honegger *et al.* 1988). Bursicon is a protein with a relative molecular mass of 30–60 (Mills & Lake, 1966; Fraenkel *et al.* 1966; Reynolds, 1977, 1983) that induces tanning of the cuticle in freshly moulted insects (Fraenkel & Hsiao, 1965; Cottrell, 1962). Its function can be tested using the 'newly emerged ligated fly bioassay' (Fraenkel & Hsiao, 1965; Seligman, 1980), in which a saline homogenate of the tissue of interest is injected into newly emerged and ligated flies. A tanning score is calculated for each test fly, the maximum score being 6 points for a fully tanned fly (Honegger *et al.* 1988). The score depends on the bursicon content of the injected homogenate. In the present

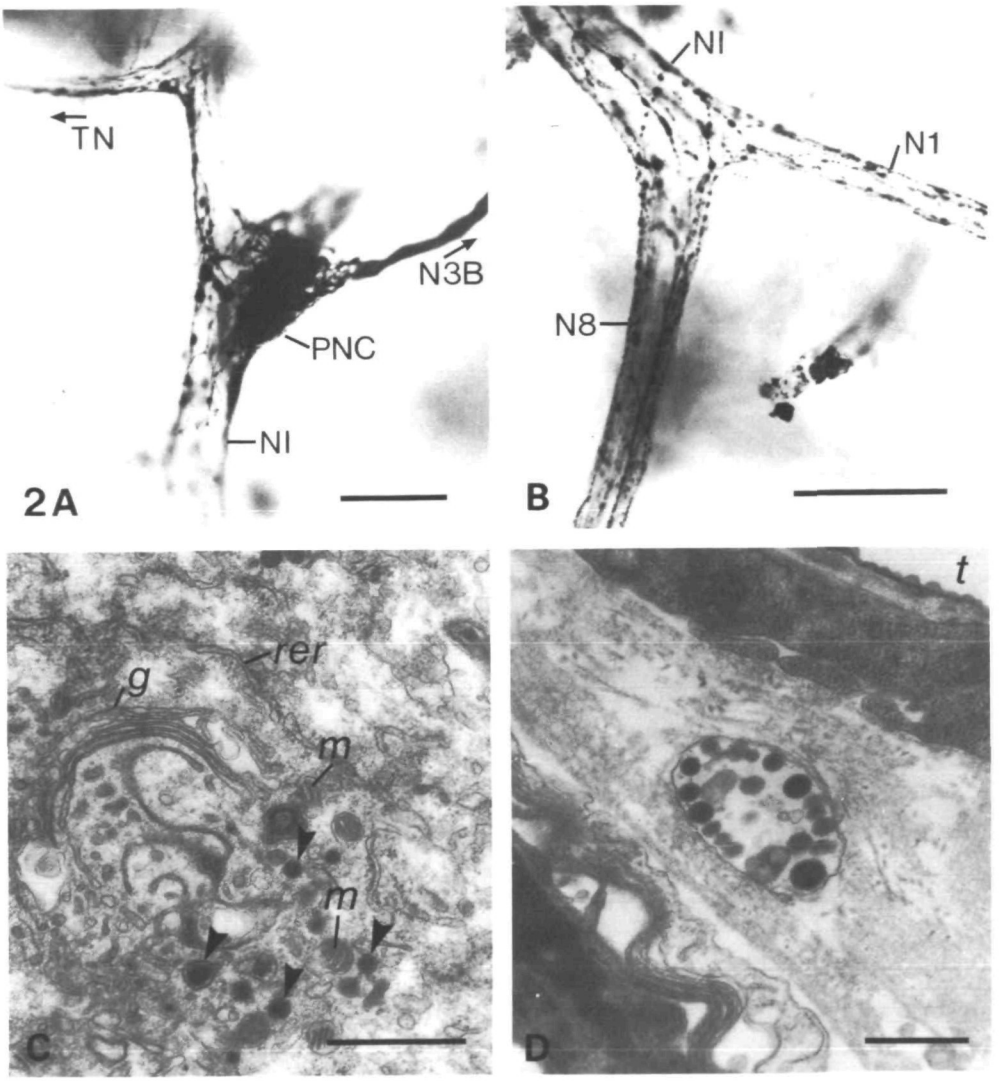

Fig. 2. Peripheral neurosecretory cells (PNC) and their projections to the prothoracic nervous system. (A) Backfill of the PNC *via* its connection to N3B (see Fig. 1). Note varicose fibres on nerve NI and the loop to the transverse nerve (TN). The somata are closely attached to NI in this preparation. Scale bar, 100 μm. (B) The same preparation as A. Note the varicose structure of stained fibres surrounding prothoracic nerve N1 and suboesophageal nerve N8 (Fig. 1). Scale bar, 100 μm. (C) Electron micrograph of the soma of one PNC. Note Golgi apparatus (*g*), rough-surfaced endoplasmic reticulum (*rer*), mitochondria (*m*) and electron-dense vesicles (arrowheads). Scale bar, 500 nm. (D) Electron micrograph of nerve 3B. One fibre profile in the sheath of the nerve contains electron-dense vesicles resembling those of the PNC cytoplasm. *t*, lumen of trachea at the surface of nerve 3B. Scale bar, 500 nm.

Table 1. *Tanning response of ligated flies after injection of homogenates of different tissue of crickets adult nervous system*

Homogenate	Mean*	S.D.	*N*
1. MN–TN	3·6	0·84	10
MN–TN+Pk	0·39, 0·9		2
I.aMN	2·18, 2·25		2
I.aMN ×2	4·06		1
2. NC	0·31	0·16	3
NC ×2	0·5, 0·47		2
NC ×10	0·14		1
3. Muscle M62 ×2	0·7, 0·58		2
4. sSOG	3·0, 2·71		2
rSOG	3·06, 3·46		2
SOG	3·78, 3·9		2
5. PNC	3·35	0·66	7
PNC+Pk	0·26, 0·41		2
6. N8(SOG) ×1·6	2·56, 3·85		2
7. N1(PTG) ×2·8	4·1, 3·63		2
8. N3(PTG)	2·96	0·66	6
9. N4(PTG)	0·2	0·2	3
N4(PTG) ×2	0·35		1
10. N5(PTG)	0·21	0·07	6
11. Saline controls	0·26	0·14	25

* Mean of the means of N separate tests with standard deviation of the means. For $N = 2$ tests the two separate scores are presented.

Each test includes one saline control. Maximum saline score was 0·5. As a standard, 10 MN–TN complexes were homogenized in 100 μl of saline; from the paired structures, nerves, PNC or muscles, 10 pairs were homogenized (Honegger *et al.* 1988).

A larger than standard concentration is indicated by a multiplication factor in column 1.

In each test 15–20 flies were injected with 5 μl of homogenate or saline. The score of each single test is the mean of the score of these flies.

MN–TN, median-transverse nerve complex; I.aMN, prothoracic anterior median nerve; NC, neck connectives; SOG, suboesophageal ganglion; sSOG, section of SOG; rSOG, remaining part of SOG after segment dissection (rSOG and SOG served as controls for sSOG); PNC, peripheral neurosecretory cells; N1–8, prothoracic (PTG) or suboesophageal (SOG) nerves, respectively, (see Fig. 1); Pk, proteinase K treatment.

study, all homogenates were made from tissue preparations of adult male and female crickets at least 14 days after eclosion.

The prothoracic MN of crickets contains fibres of two cell clusters in the posteriolateral region of the prothoracic ganglion (Honegger *et al.* 1988), axons of four motoneurones in the suboesophageal ganglion innervating the neck muscles M62 (Honegger *et al.* 1984) and fibres of the PNC.

No tanning activity could be detected in the saline homogenates of ganglion sections containing the two prothoracic cell clusters (mean score 0·26, $N = 4$ tests with 20 flies per test; mean score of saline controls 0·1; Honegger *et al.* 1988). In

locusts, homologous cell populations have been shown to be FMRFamide- and bovine pancreatic polypeptide (BPP)-immunoreactive (Myers & Evans, 1985*a*,*b*). The negative result with homogenates of the posterior lateral cell clusters corresponds with our observation that FMRFamide (10^{-4} to 10^{-8} mol l^{-1}, Sigma) has no tanning activity in the bioassay (score equals saline controls). Thus, the posterior lateral cells of the prothoracic ganglion do not contribute to the bursicon-like activity of the MN.

The axons of two of the four suboesophageal M62 motoneurones with their cell bodies in the posteriolateral labial neuromere (Fig. 1) descend through the neck connectives to the prothoracic ganglion, where they turn through 180°, without branching, and leave through the prothoracic anterior median nerve (I.aMN; Fig. 1) to proceed through both transverse nerves towards muscles M62. The axons of the other two motoneurones with their somata at the border between the labial and maxillary neuromeres project to the same muscles *via* the suboesophageal posterior median nerve (S.pMN; Fig. 1) and the transverse nerves (Honegger *et al.* 1984). We tested for bursicon activity in homogenates of the I.aMN, the neck connectives, the muscles M62 and in sections of both sides of the posterior suboesophageal ganglion which contain the somata of the two M62 motoneurones. Since the cell body positions are variable, the ganglion sections had to be fairly large. They were dissected with one cut, made slightly anterior and parallel to nerve N5 and a second cut perpendicular to the first extending to the lateral edge of the neck connective, and contained roughly 600–650 somata each (numbers calculated from 10 μm transverse sections stained with ethyl gallate).

Homogenates of I.aMN caused tanning in test flies but homogenates of the neck connectives and muscles M62 did not (Table 1). These results indicate that the two M62 motoneurones with the more posterior somata and axons projecting through the neck connectives can be ruled out as a source of bursicon-like material since homogenates of these as well the neck connectives of the I.aMN and of muscles M62 should then have caused tanning. Suboesophageal ganglion sections contain bursicon-like activity (Table 1). Therefore, the other two motoneurones with more anterior somata may contribute to the bursicon-like activity found in the median-transverse nerve complex.

Saline homogenates of the prothoracic PNC of adult crickets show bursicon-like activity (Table 1). In addition, homogenates of all the nerves covered with varicose fibres from the PNC (Fig. 1) show bursicon-like activity (Table 1). Since the cobalt histology shows the PNC net to be of comparable density on all nerve surfaces, nerves with a small diameter, such as N8(SOG) and N1(PTG), must carry less bursicon-like material than nerves with a large diameter, such as N3(PTG). In these tests, therefore, concentrations of nerve homogenates were normalized with respect to the surface area of N3(PTG) (Table 1). Homogenates of the prothoracic nerves N4 and N5, which do not carry PNC fibres, had no detectable tanning activity even when doubly concentrated homogenates were injected (Table 1; N5 is the prothoracic nerve with the largest diameter, N4 has an equal diameter to N3).

Although the structure in which the PNC are embedded is tiny, homogenates of the PNC of standard concentration had a high tanning score (Table 1). This indicates strongly that the bursicon activity is intrinsic to the PNC and does not originate from fibres of unknown central neurones innervating the PNC. Our anatomical results support this conclusion. First, cobalt stainings of prothoracic nerves never revealed any axon collaterals projecting to the PNC. Second, electron micrographs of the PNC cluster showed only few fibres embedded in the thin connective tissue sheath surrounding them. These few profiles, derived from unknown efferent neurones, are very unlikely to account for the high bursicon activity. In addition, these fibres contain vesicles of the same type as the PNC cytoplasm, suggesting that they originate from the PNC.

The bursicon-like activity of the PNC and of the MN is completely abolished after 3 h of incubation of homogenates with proteinase K (5 mg ml^{-1}, Sigma) at 37 °C (Table 1). Thus, the tanning activity of the prothoracic PNC in crickets is associated with at least one peptide.

Our results show for the first time that a distinct group of prothoracic peripheral neurosecretory cells contains a bursicon-like neuropeptide. Bioassays combined with cobalt stains indicate that it is also present in fibres of homologous PNC of the mesothoracic segment. Bursicon-like activity, has been demonstrated in ganglia of the central nervous system of various insects (Mills & Lake, 1966; Taghert & Truman, 1982*a*; Vincent, 1972), including crickets (Honegger *et al.* 1988; and this report). In *Manduca sexta*, bursicon released shortly after eclosion seems to be produced by four central neurones of the abdominal ganglia and released *via* abdominal NHO (Taghert & Truman, 1982*b*). It remains to be investigated whether the centrally and peripherally derived bursicon-like proteins are identical.

Considerable amounts of a bursicon-like substance must be synthesized in the PNC of adult crickets, although its function in adult insects is unknown. It is possible, however, that it regulates the tanning of growing cuticle in adults in the same way as in larvae. In adult locusts, the apodemes and other parts of the endocuticle show daily growth layers (Neville, 1967), and the endocuticle continues to grow for up to 3 weeks after adult emergence (Andersen, 1973). In addition, bursicon could be used in adult insects during the process of wound repair when epidermal cells secrete new cuticle (Neville, 1975).

We have shown that a bursicon-like peptide is produced by nerve cells lying in the periphery of the nervous system. The activity of nerve cells in the periphery of the nervous system, which are poorly insulated from the haemolymph, may be directly controlled by circulating levels of hormones and ions. The resulting secretion of bursicon from the fibres of these nerve cells may be directed not only into the exterior haemolymph but also into the interior medium, i.e. the central nervous system, thereby modulating its activity.

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