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THE DOPAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF LOCUST AND COCKROACH SALIVARY NEURONES

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Summary

The salivary glands of the cockroach and locust are innervated primarily from two pairs of motoneurones, designated SN1 and SN2, in the suboesophageal ganglion. Intracellular cobalt fills and subsequent silver intensification were used to reveal the morphology of these cells in both species.

Fluorescent microscopy, following treatment of the ganglion with glyoxylic acid, showed that in both species only the SN1 neurones contained catecholamines. A radioenzymatic assay for dopamine, performed on the locust SN1 neurones, confirmed that this catecholamine was present.

A radioenzymatic assay for 5-hydroxytryptamine (5-HT), performed on both pairs of salivary neurones in the locust, revealed small quantities of this amine in the SN2 neurones, but no significant amount in the SN1 neurones. In the cockroach, 5-HT was assayed in the SN2 neurones only. In contrast to the locust, however, the 5-HT content of these cells was not significantly above that of control cells taken from other ganglia.

These observations demonstrate that only the SN1 neurones are the source of the catecholaminergic fibres investing the locust and cockroach salivary glands. The difference in neurotransmitter content between the SN1 and SN2 neurones suggests that these neurones have separate functions in the control of salivary secretion.

Introduction

Insect salivary glands, particularly those of locusts, cockroaches and blowflies, have proved to be valuable preparations in which to study peripheral monoamin-

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ergic neurotransmission. In blowflies, the salivary glands are not innervated and are controlled by 5-HT transported in the haemolymph (Berridge and Prince, 1971; Hansen Bay, 1978). By contrast, in locusts and cockroaches the glands are innervated primarily from the suboesophageal ganglion, *via* the bilaterally paired nerves 7 (the salivary nerves; Whitehead, 1971; Altman and Kien, 1979). Cobalt backfills through these nerves have demonstrated that each contains the axons of a pair of motoneurones from the suboesophageal ganglion, designated SN1 and SN2 (Altman and Kien, 1979; Fleming, 1986). Cockroach salivary glands, but apparently not locust glands, are also innervated by branches from the stomatogastric nerves (Whitehead, 1971; Bowser-Riley, 1978).

Falk–Hillarp fluorescence histochemistry, immunocytochemistry and radioenzymatic and HPLC assays have revealed the presence of both dopaminergic and 5-HT-containing nerve fibres in cockroach and locust salivary glands (Klemm, 1972; Bland *et al.* 1973; Fry *et al.* 1974; Mitchell and Williams, 1981; Tyrer *et al.* 1984; Baines *et al.* 1989). A large quantity of pharmacological data also support a neurotransmitter role for these amines in the glands of cockroaches (reviewed by House and Ginsborg, 1985; see also Evans and Green, 1990*a*,*b*) and locusts (Baines and Tyrer, 1989).

In cockroaches, at least some of the 5-HT-containing fibres found in the salivary gland originate from the stomatogastric nerve (Davis, 1985). By contrast, immunocytochemical evidence suggests that the source of the 5-HT fibres in locusts is the suboesophageal salivary cells (Tyrer *et al.* 1984). As regards the dopaminergic innervation of the salivary glands, the source of these fibres in cockroaches is the suboesophageal ganglion (Baker and Pitman, 1989). Which of the salivary neurones contains this amine, however, is unknown. There have been no reports on the source of the dopaminergic innervation in locust salivary glands.

To provide information on the origin of the aminergic innervation of the salivary glands in locusts and cockroaches the transmitter content of the suboesophageal salivary neurones was analysed using glyoxylic acid histochemistry and biochemical assays.

Materials and methods

The suboesophageal ganglion was removed from the insect, desheathed and placed in an oxygenated saline bath. For locusts (*Schistocerca gregaria* Forskål) the saline contained 128 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 3 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} Tes buffer, pH 7.4, whereas for cockroaches [*Periplaneta americana* (L.)] the saline contained 214 mmol l^{-1} NaCl, 3.1 mmol l^{-1} KCl, 9.0 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} Tes buffer, pH 7.2. Microelectrodes were filled with either 4 % Procion Navy Blue or 100 mmol l^{-1} hexamminecobaltic chloride and had a resistance of 30–50 MΩ.

With the exception of the cockroach SN1 neurones, the locust and cockroach salivary cells could be readily identified in the ganglion by their large size relative to the surrounding neurones. In most cases identification of the cells was

additionally confirmed by the antidromic action potential evoked by stimulation of nerve 7.

Unlike the SN2 salivary cells, the cell bodies of cockroach SN1 neurones in the intact ganglion are covered by the bodies of several large motoneurones that had to be removed before the SN1 neurones could be impaled. This meant that the SN1 cells were relatively difficult to find and impale compared to the other salivary neurones.

Radioenzymatic assays

Salivary cells were first functionally identified and then marked by intracellular injection of a small amount of Procion Navy Blue. The ganglion was then transferred to another dish of saline and the cells were dissected free. The salivary neurones were cleaned of adhering cells and transferred, using a small wire loop, into either 25 μl of 0.1 mol l^{-1} perchloric acid (for dopamine, DA, assay) or 25 μl of 0.05 mol l^{-1} phosphate buffer (for 5-HT assay). Cell bodies of nearby motoneurones were isolated in a similar manner and used as controls. Samples were stored in liquid nitrogen until assayed.

The DA and 5-HT contents of the salivary cells were determined by radioenzymatic assay. DA was assayed using the procedure described by Evans *et al.* (1985). The assay for 5-HT was performed using the method described by Hussain and Sole (1981), with the exception that the pineal glands from sheep, rather than cows, were used as the source of hydroxyindole-*o*-methyltransferase. Twice background sensitivity limits were approximately 15–20 fmol for both these amines. The detection limits for DA and 5-HT were additionally improved by assaying a pooled sample of 3–13 salivary cells.

Glyoxylic acid fluorescence histochemistry

Catecholaminergic neurones and processes were visualized using a whole-mount glyoxylic acid fluorescence technique modified from that used by Baker and Pitman (1989).

The ganglia were dissected from the insect, desheathed and placed in a glyoxylic acid solution (360 mmol l^{-1} glyoxylic acid, 100 mmol l^{-1} KH_2PO_4, 80 mmol l^{-1} sucrose, pH 7.4). After being left in this solution for about 1 h the ganglia were placed on a depression slide and put in a freeze-drier precooled to −40 °C. The tissue was not frozen with liquid nitrogen since this seemed to give poorer results. When the ganglia had dried they were removed from the freeze-drier and heated in an oven for 6 min at 95 °C. The ganglia were then allowed to cool before being cleared in xylene, mounted in D.P.X. and examined with a Zeiss fluorescent microscope (primary filter 420 nm, secondary filter 530 nm).

Results

Morphology

The morphology of the salivary cells was revealed by intracellular injection of

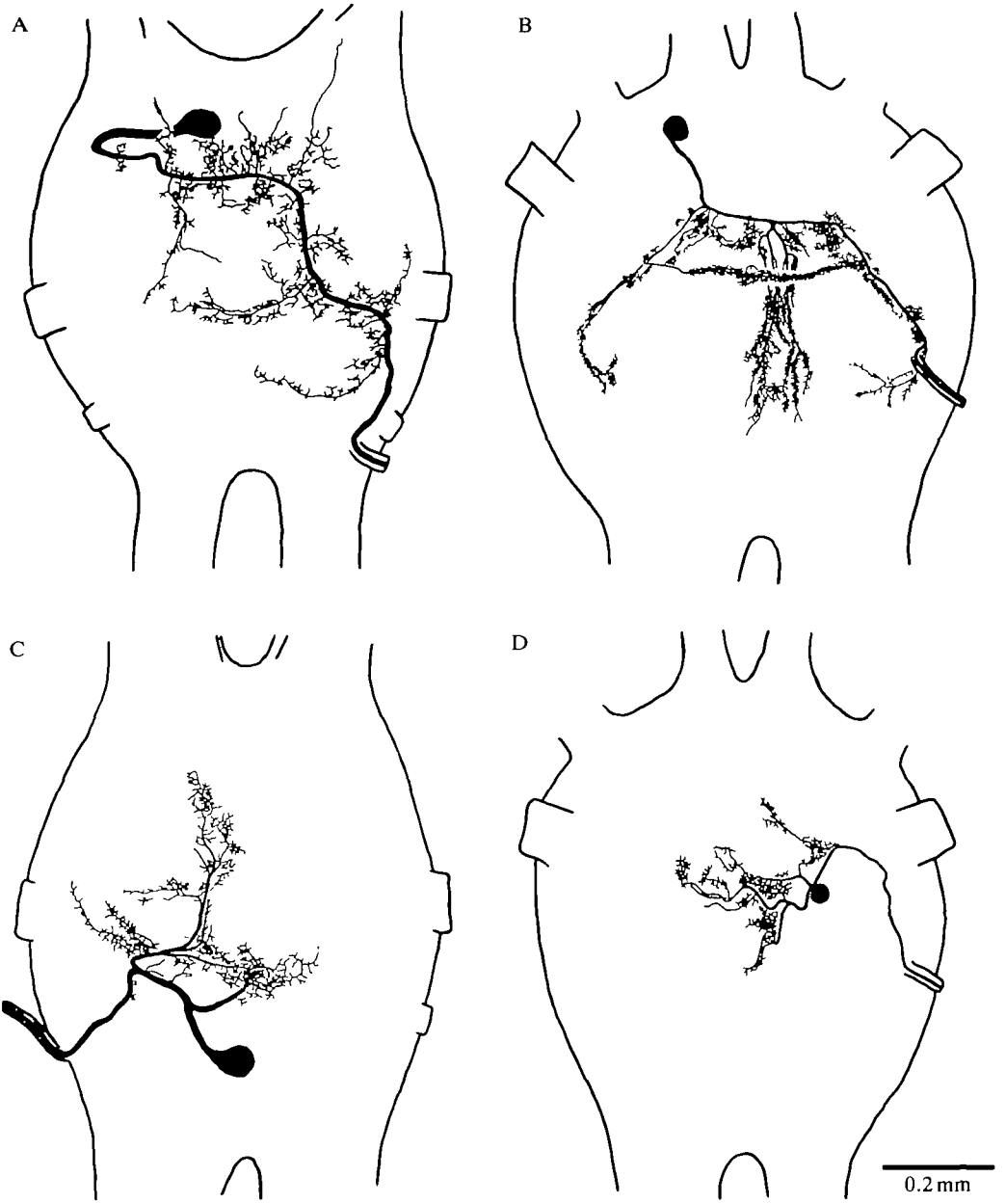

Fig. 1. *Camera lucida* drawings of salivary neurones, as revealed by cobalt filling *via* the cell body, of (A) locust SN1, (B) cockroach SN1, (C) locust SN2, (D) cockroach SN2 cells.

hexamminecobaltic chloride, *via* the soma, followed by treatment with ammonium sulphide and silver intensification (Pitman *et al.* 1972; Pitman, 1979).

The cell bodies of the SN1 neurones are located in the anterior part of the ganglion (Fig. 1A,B). In locusts they are situated on the dorsal surface of the

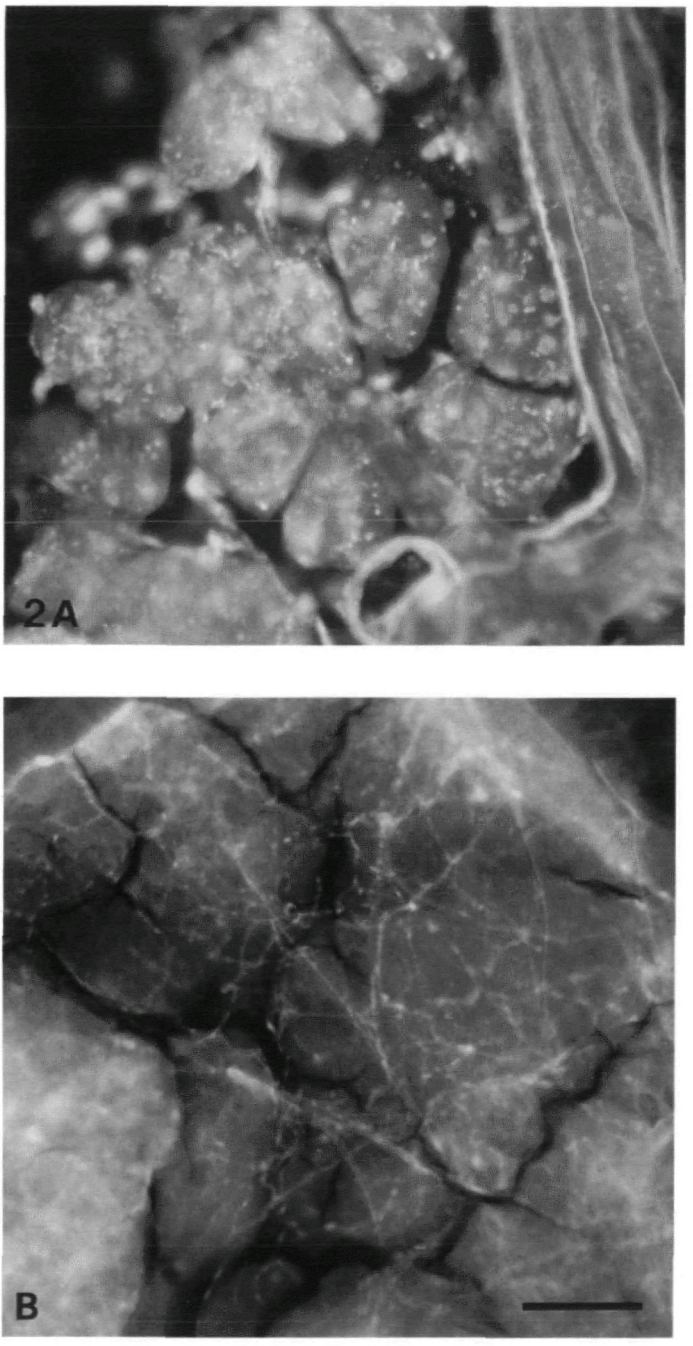

Fig. 2. Salivary glands of (A) locust and (B) cockroach after glyoxylic acid treatment showing the dense network of catecholaminergic fibres (blue-green). Scale bar, 0.2 mm.

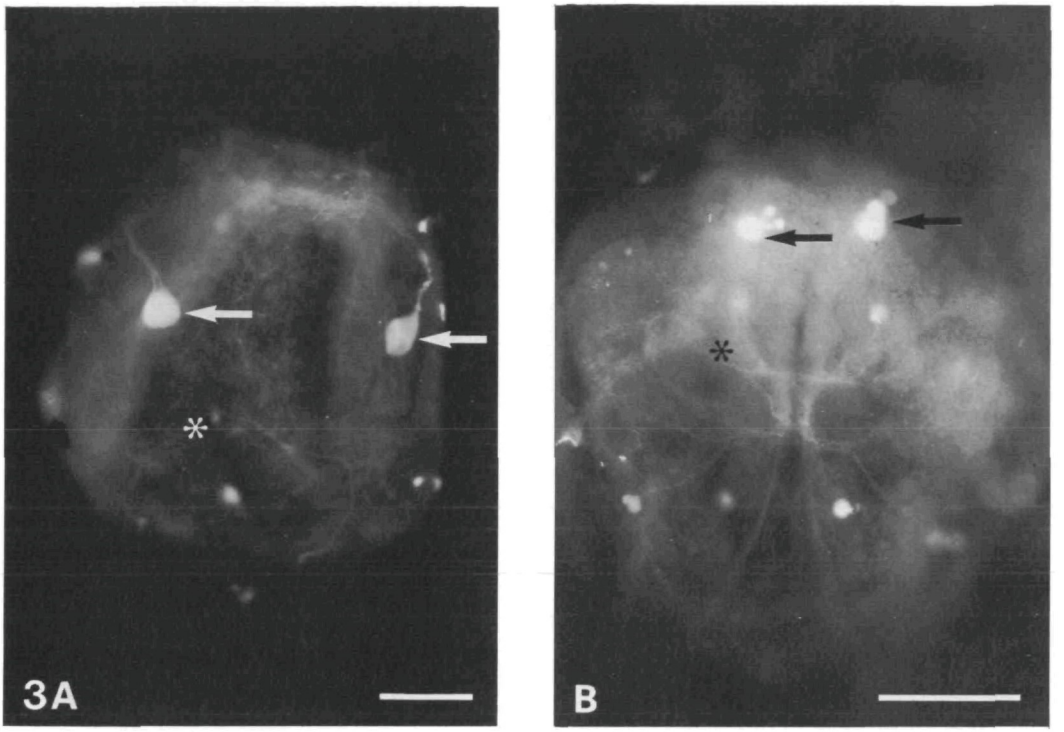

Fig. 3. Suboesophageal ganglion of (A) locust and (B) cockroach after glyoxylic acid treatment. The SN1 neurones are arrowed. The approximate position of one member of the pair of SN2 neurones, which unlike the SN1 neurones are not fluorescent, is marked by the asterisks. Sale bars, 0.2 mm.

ganglion, whereas in cockroaches the SN1 neurones are located on the ventral surface. In both locusts and cockroaches the axon of SN1 passes into the contralateral salivary nerve. The SN2 neurones in locusts and cockroaches are more posteriorly located (Fig. 1C,D). In both species they are situated on the ventral surface of the ganglion and send an axon into the ipsilateral salivary nerve.

Fluorescence histochemistry

A network of green-fluorescent fibres is visible in locust and cockroach salivary glands after treatment with glyoxylic acid (Fig. 2A,B). Glyoxylic acid treatment of both locust and cockroach suboesophageal ganglia revealed two green-fluorescent cells in the anterior part of the ganglion, corresponding in size and position to the SN1 neurones (Fig. 3A,B). In many preparations the axons of these cells could be traced all the way to the salivary nerve, confirming their identity (Fig. 4A,B).

No fluorescence was visible in the locust and cockroach SN2 neurones and thus the SN1 neurones are presumed to be the sole source of the catecholaminergic fibres seen in the salivary glands. Although several other fluorescent neurones are present in the suboesophageal ganglion, it is unlikely that these also innervate the

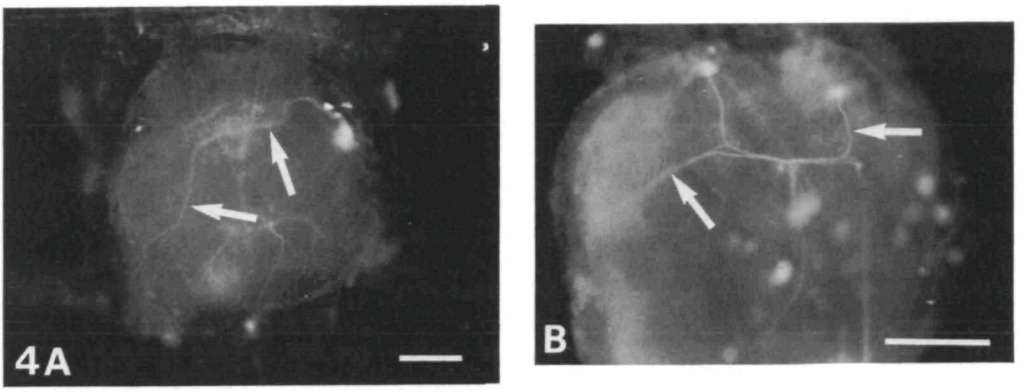

Fig. 4. Suboesophageal ganglion of (A) locust and (B) cockroach to show the axon of SN1 (arrowed) within the neuropile. Scale bars, 0.2 mm.

Table 1. *Amine content of salivary neurones*

Amine	Cell	Replicates	Amount (fmol/soma)
DA	Locust control	4	8.7±5.2
DA	Locust SN1	4	86.3±25.8*
5-HT	Locust control	8	1.0±0.5
5-HT	Locust SN1	4	0.3 ± 1.4^{NS}
5-HT	Locust SN2	8	6.9±2.3**
5-HT	Cockroach control	4	0 ± 0.3^{a}
5-HT	Cockroach SN2	5	0.2 ± 0.5^{NS}

Values are means (±S.E.M.) of the number of replicates indicated.

Each replicate consisted of a pooled sample of 3–6 cells in the case of dopamine assays and 4–13 cells in the case of 5-hydroxytryptamine assays.

NS, not significantly different from control; * $P<0.05$ *versus* control; ** $P<0.01$ *versus* control; [a] mean value was slightly below blank values (but within the range of experimental error).

DA, dopamine; 5-HT, 5-hydroxytryptamine.

salivary gland since cobalt backfills have demonstrated that SN1 and SN2 are the only suboesophageal neurones sending axons through the salivary nerve (Altman and Kien, 1979; J. R. Baker and R. M. Pitman, unpublished observations).

Radioenzymatic assays

Radioenzymatic assays for catecholamines confirmed that the green fluorescence in locust SN1 neurones resulted from the presence of DA in these cells (Table 1). 5-HT was assayed in locust SN1 and both locust and cockroach SN2 neurones. Neither cockroach SN2 nor locust SN1 neurones contained levels of 5-HT significantly above the controls. However, a small amount of 5-HT was found in locust SN2 neurones.

To determine the volume, and thus the concentration, of biogenic amines in the locust salivary neurones five locust SN1 neurones and six locust SN2 neurones were filled with Procion Navy Blue dye, fixed in formaldehyde, dehydrated, cleared and the cell diameter measured under a microscope. The concentration of DA in the SN1 neurones was calculated to be approximately 2.5 mmol l^{-1} and the concentration of 5-HT in the SN2 neurones was calculated to be 0.24 mmol l^{-1}.

Discussion

Locust

After glyoxylic acid treatment, locust SN1 neurones showed a green fluorescence and are thus presumably the source of the network of green-fluorescent, varicose fibres in the salivary glands. The green fluorescence in these cells is characteristic of catecholamines, although it is not possible to determine which catecholamine is present without microspectrofluorometric analysis (see Bjorklund and Falk, 1973). HPLC analysis of the locust salivary glands by Baines *et al.* (1989) revealed the presence of both DA (24 ng/gland) and noradrenaline (NA) (62 ng/gland), although the latter amine was also present in control tissues (flight muscle) not known to have a noradrenergic innervation. In the present study, however, radioenzymatic assay of the locust SN1 neurones demonstrated that they contain DA (86 fmol/cell).

In contrast to the SN1 neurones, the SN2 neurones in locusts showed no catecholamine fluorescence after glyoxylic acid treatment. Radioenzymatic assay revealed the presence of 5-HT in the cell bodies of these cells (6.9 fmol/cell). The 5-HT detected by Baines *et al.* (1989) in the locust salivary gland (116 ng/gland) thus presumably originates from these cells, although the amount of 5-HT in the SN2 neurones is surprisingly low when compared with the amount of DA in the SN1 neurones. The relatively low concentration of 5-HT in the soma of the SN2 neurones can explain the absence of a yellow, 5-HT-induced fluorescence in the cells following glyoxylic acid treatment. The concentration of 5-HT in these cells is only a fraction of that found in leech Retzius cells (approximately 7 mmol l^{-1}; Wallace, 1981), which fluoresce brightly in glyoxylic-acid-treated preparations. Thus, in insect serotonergic neurones either 5-HT synthesized in the soma is rapidly transported out into the neurites or synthesis of 5-HT is mainly restricted to axons and/or nerve terminals rather than the cell body.

No 5-HT was detected in the SN1 neurones in the locust. This finding contrasts with the immunocytochemical results obtained by Tyrer *et al.* (1984), who found that both pairs of salivary neurones reacted with antibodies to 5-HT. However, the SN1 neurones showed a weaker and more variable immunoreactivity than the SN2 neurones (N. M. Tyrer, personal communication). It is thus possible either that the SN1 neurones contain a concentration of 5-HT that was too small to be detected by the radioenzymatic assay or that the 5-HT antibodies were cross-reacting with the DA, or its precursors, in these neurones.

In addition to a difference in the transmitter employed by SN1 and SN2 there is

a difference in the physiology of these cells. Extracellular recordings from the salivary nerve have shown that, when the locust is not feeding, the spike frequency of SN2 is about 20 times that of SN1. During feeding the activity of both neurones increases, but the increase in SN1 is relatively much greater (Baines and Tyrer, 1989). These two pairs of neurones also differ in their response to activity in the transverse nerve, branches of which terminate in the salivary gland. Stimulation of this nerve produces a large increase in the firing rate of SN1, but has no effect on the firing rate of SN2 (Baines and Tyrer, 1989).

Cockroach

In cockroaches, as in locusts, only the SN1 neurones fluoresce after treatment with glyoxylic acid. The green fluorescence almost certainly results from DA, since microspectrofluorometric analysis and radioenzymatic assay performed on the salivary glands has indicated that they contain DA and that NA is absent (Klemm, 1972; Fry *et al.* 1974).

The SN2 neurones in the cockroach showed no catecholamine fluorescence following glyoxylic acid treatment and, in contrast to locust SN2 neurones, radioenzymatic assay indicated that 5-HT levels did not differ significantly from control neurones. This latter finding is supported by the absence of cells corresponding to the SN1 or the SN2 neurones in maps of the 5-HT immunoreactivity in the cockroach suboesophageal ganglion made by Bishop and O'Shea (1983). Furthermore, the salivary nerve in this species contains only one 5-HT immunoreactive axon, which is one of several small-diameter axons in this nerve and is not the axon of SN1 or SN2 (Davis, 1985).

One possible neurotransmitter candidate for SN2 neurones is octopamine. This amine is present (in similar amounts to DA) in the salivary glands of *Nauphoeta cinerea* Olivier (Mitchell and Williams, 1981) and when applied to the salivary glands it potentiates neurally evoked secretory potentials (Bowser-Riley and House, 1976). This effect of octopamine on neurally evoked secretory potentials is similar to its potentiating action on excitatory potentials at the neuromuscular junction of the locust (Evans and O'Shea, 1977; O'Shea and Evans, 1979).

Interestingly, an electron microscope study by Maxwell (1978) demonstrated the presence of at least two ultrastructurally distinct types of axon terminal (designated type A and type B) in the cockroach salivary gland. Several histochemical properties suggest that only the type A axon terminals contain catecholamines (Maxwell, 1980). These terminals thus presumably correspond to those of the SN1 neurones. The type B terminals probably correspond either to the axon terminals of the SN2 neurones or to axon terminals from the stomatogastric nerve. Alternatively, they may be processes from the cells responsible for the small-diameter axons present in the salivary nerve.

The similarities in position and axonal projections suggest that salivary neurones in cockroaches are homologous with those in locusts. However, although the SN1 neurones in the two species both appear to use DA as a transmitter, the SN2 neurones seem to have diverged in their transmitter content.

The difference in the transmitters used by SN1 and SN2 suggests that these neurones have separate functions in controlling salivary secretion. In locusts, this proposal is supported by the observation that the cells differ in their spontaneous firing rate and respond differently during feeding. The behaviour of the cockroach SN1 and SN2 neurones has not been investigated, although the demonstration of a similar difference in properties would obviously be of value in the context of the findings reported here.

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