On the Aminergic Innervation of Locust
(\textit{Locusta migratoria}) Salivary Glands

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
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Abstract

This study is a characterization of the aminergic innervation of the salivary glands of the locust, Locusta migratoria. The salivary glands of Locusta are innervated via one pair of tyrosine hydroxylase-like positive cells (SN1) and one pair of serotonin-like positive cells (SN2) whose somata are located within the suboesophageal ganglion. Serotonin and dopamine induce dose-dependent elevations of cyclic AMP levels in the salivary glands. The rank order of potencies of serotonergic and dopaminergic antagonists suggest the presence of a receptor similar to vertebrate 5-HT₂ and vertebrate D₁ receptors. The increases in cyclic AMP levels are mimicked by neural stimulation of the salivary nerve which can be partially inhibited by the dopaminergic antagonist SCH-23390, and by the serotonergic antagonist spiperone. Neural stimulation of the salivary nerve leads to time- and frequency-dependent elevations of cyclic AMP levels in the glands that can be partially inhibited by SCH-23390.

Uptake transporters for serotonin and dopamine are associated with the salivary glands. The uptake of [³H]serotonin is Na⁺-dependent and is composed of high- and low-affinity components. The uptake of [³H]dopamine is Na⁺-independent. The efflux of [³H]serotonin and [³H]dopamine from the salivary glands is Ca²⁺-dependent and occurs in response to stimulation of the salivary nerve and to treatment with high-K⁺ saline.

Neural stimulation of the maxillary nerve on the SOG results in phase locked SN1 activity at low stimulatory frequencies but not at high. Perfusion of desheathed suboesophageal
ganglia with acetylcholine, cholinergic agonists, and octopamine leads to enhanced activity of either SN1 or SN2, or both. Perfusion with either dopamine, serotonin, glutamate or GABA has no apparent effect on salivary neuron activity.

A comparative study of salivary gland innervation in the cockroach, *Periplaneta americana*, and the stick insect, *Carausius morosus*, was performed. Tyrosine hydroxylase-like immunoreactivity of the SOG of *Periplaneta* and *Carausius*, reveals a dopaminergic SN1 pair in both animals. Serotonin-like immunoreactivity of the SOG and salivary glands of *Carausius* reveals a serotonergic SN2 pair. A phylogenetic analysis using neuroanatomical and neurophysiological data for *Locusta, Carausius* and *Periplaneta*, suggest that Phasmatodea and Orthoptera are sister taxa.
**Résumé**

Cette étude est une caractérisation de la innervation aminergique des glandes salivaires du criquet, *Locusta migratoria*. Les glandes salivaires de *Locusta* sont innervées par une paire de cellules positives à la sérotonine dont les stomata sont situés dans le ganglion sous-oesophagien. La dopamine et la sérotonine déclenchent une augmentation de cAMP dans les glandes salivaires. L'ordre de puissance des drogues antagonistes de la dopamine et la sérotonine suggèrent que ces récepteurs sont similaires aux récepteurs D₁ et 5-HT₂ des vertébrés. Les augmentations de cAMP sont imitées par la stimulation du nerf salivaire qui peut être partiellement bloquées par l'antagoniste dopaminergique SCH-23390, et par l'antagoniste sérotonergique spiperone. La stimulation du nerf salivaire provoque une augmentation de cAMP dans les glandes salivaires qui dépend du temps et de la fréquence et peut être partiellement bloquée par le SCH-23390.

Les glandes salivaires possèdent des mécanismes de capture de la dopamine et de la sérotonine. La capture de la [³H]sérotonine se fait grâce à des mécanismes à haute et basse affinité et est sensible au Na⁺. La secretion de la [³H]sérotonine et de la [³H]dopamine des glandes salivaires est sensible au Ca²⁺ et est stimulée par le nerf salivaire et par une solution saline à haute teneur en K⁺.

La stimulation du nerf maxillaire dans les SOG aboutit à une activité phase bloquée sensible aux fréquences basses, pas aux fréquences hautes. On perfuse les ganglion sous-oesophagien dégainés avec l'acétylcholine, les agonistes cholinergique, et l'octopamine. Ces interventions
aboutissent à une activité intensifiée du SN1 ou SN2 ou a les deux. Il n’y a pas d’effet apparent de l’activité de neuro-salivaire si on perfuse le nerf avec la dopamine, la sérotonine, le glutamate, ou le GABA.

Une étude comparative de l’innervation de la glande salivaire du cafard, *Periplaneta americana*, et de l’insecte branchette, *Carausius morosus* fut effectuée. L’immunoreactivité à la tyrosine hydroxylase de la SOG de *Periplaneta* et *Carausius*, était présente dans un paire de neurones dopaminergiques SN1 dans les deux animaux. L’immunoreactivité à la sérotonine de la SOG et des glandes salivaires de *Carausius* est localisée dans un paire sérotonergique SN2. Une analyse phylogénique qui utilise les données neuroanatomiques et neurophysiologiques de *Locusta*, *Carausius* et *Periplaneta*, suggère que les Phasmatodea et les Orthoptera sont apparentés.
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Organization of the Thesis

Chapter II was published in the Journal of Insect Physiology (Ali D.W., Orchard I. and Lange A.B. (1993) J. Insect physiol. 39, 623-632.), and was jointly authored with Dr. Angela B. Lange and Dr. Ian Orchard. I performed several of the immunohistochemical experiments and the cyclic AMP determinations while Dr. Lange performed various immunohistochemical experiments along with the HPLC analyses. Chapter III was published in the journal, Biogenic Amines Ali D.W. and Orchard I. (1994) Biogenic Amines 10, 195-212). Chapter IV was published in the Journal of Experimental Biology (Ali D.W. and Orchard I. (1996) J. exp. Biol. 199, 699-709). Chapter V is being prepared for publication. Chapter VI was published in the journal, Cell and Tissue Research (Ali D.W. and Orchard I. (1996) Cell Tissue Res. 285, 453-462). Chapter VII was published in the Journal of Insect Physiology (Elia A.J., Ali D.W. and Orchard I. (1994) J. Insect Physiol. 40, 671-683). All of the experiments in Chapter VII were performed equally between myself and Dr. Andrew J. Elia, although Dr. Elia wrote the majority of the manuscript. Chapter VIII is being prepared for publication. All of the neurophysiological experiments were performed by myself, but Dr. D. Christopher Darling (Royal Ontario Museum, Toronto) performed all of the phylogenetic analyses since his expertise in this field was required.

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Permission was granted from the respective publishers to reprint Chapters II, III, IV, VI and VII.
General Introduction

Salivary glands are glands associated with nutrient intake whose secretions are generally involved in the digestion and lubrication of food. Salivary secretions vary within the insect class and their composition is linked to feeding and digestion. For instance, the saliva of many insects contains digestive enzymes, while in others it may contain anticoagulants or may be used primarily for lubrication. In addition, the salivary glands of some insects (Lepidoptera) produce silk at certain stages of their life cycle (Kafatos 1968). Insect diets are of a wide variety and include dead and decaying matter, plant tissue, other insects and blood (Slansky and Scriber 1985). Feeding mechanisms (Smith 1985), salivary gland structure and salivary gland secretions have therefore been modified to meet the different needs and feeding habits of insects.

Salivary, or labial glands, appear to have arisen from those tissues which produce secretions involved in food intake in invertebrates such as insects, annelids and molluscs. The term labial, arises because of the region in the head cavity to which the ducts project. Given the salivary role of the secretions from the labial glands, the term salivary glands will be used throughout.

Annelids and Molluscs

The salivary glands of other groups of invertebrates have been investigated to some degree. Those of the giant Amazonian Leech, *Haementeria ghilianii*, are composed of thousands of cells, each of which extends a single ductule into the proboscis. The individual gland cells are capable of supporting action potentials, but are themselves not innervated. The salivary duct cells are innervated from a branch of the stomatogastric nervous system which enters the proboscis near its tip to innervate the ductules. Stimulation of the stomatogastric salivary nerve elicits electrical activity in the ductules which travels towards the salivary gland cell bodies (Wuttke *et al.*, 1989). The cell bodies support calcium-dependent, overshooting action potentials which initiate a secretory response from the glands (Wuttke *et al.*, 1989). Application of serotonin to the ductules and cell bodies results in secretion of saliva and an increase in excitability of the somata.
Similarly, increased levels of serotonin in the haemolymph of the leech, *Hirudo medicinalis*, are capable of increasing salivation in this animal (Marshall and Lent, 1988). In addition, the presence of serotonergic axons in the proboscis sheath of glossiphonid leeches (Marshall and Lent, 1988) lends additional support towards a possible role for serotonin as a salivary neurotransmitter in leeches.

The salivary glands of the gastropod mollusc, *Helisoma trivolvis*, are innervated via a salivary nerve which projects from the main oesophageal trunk. The glands are composed of two main tubes with acinar outcroppings (Kater *et al.*, 1978a) that are extensively electrically coupled, and are thus capable of propagating action potentials. The glands are innervated from neurons in the buccal ganglion, which, in *Lymnaea stagnalis*, stain positively for myomodulin and small cardioactive peptide (SCP) (Santama *et al.*, 1994), suggesting a possible salivary gland neurotransmitter role for these peptides. However, it has been postulated that acetylcholine is a possible salivary neurone effector transmitter in the salivary glands of the snails *Philine aperta*, *Limax maximus*, and *Helix aspersa* (Barber 1983; Barry and Gelperin 1982; Osborne 1977).

Perhaps the most intricately innervated salivary gland system in Molluscs is found in the octopods where the ducts may be innervated by up to 10,000 axons and the glands by 30,000 axons (Young 1965a, b). The paired salivary duct nerves originate from the subradular ganglia whereas the gland nerves arise from the superior buccal lobe of the brain (Young 1965a) and contain serotonergic and noradrenergic fibres (Barlow *et al.*, 1974; Arluison and Ducros 1976). Stimulation of the salivary nerve elicits secretion of a saliva rich in serotonin, noradrenaline, dopamine, octopamine, tyramine, various enzymes and a cephalotoxin (Ghiretti 1950, 1953, 1960; Barlow *et al.*, 1974).

**Insect Salivary Glands**

Insect salivary glands are of two main types, acinar as in locusts and cockroaches, and tubular as in the blowfly. Tubular glands are long, coiled tubes which extend into the thorax and abdomen and open at their proximal end into the preoral cavity. The tubular epithelial layer is one cell thick and is covered by a basement membrane (House and
Acinar glands have a greater degree of structural complexity. The fluid secreting region or acini, produce and secrete saliva into a series of salivary ducts which eventually join to form main ducts which open into the preoral cavity (House and Ginsborg 1985).

Salivary glands may be innervated from several different sources. Many insects have a salivary nerve that projects from the suboesophageal ganglion (SOG), but several species (*Periplaneta americana* and *Rhodnius prolixus*) are also equipped with a salivary nerve which projects from the stomatogastric nervous system (Davis 1985; Baptist 1941). In addition, projections from the median nervous system may also exist, as it does in *Locusta migratoria* (Myers and Evans 1985). Tubular glands are not innervated to the same extent as acinar glands; for example, in the moth *Manduca sexta*, the glands of the adult are innervated from the stomatogastric nervous system (Robertson 1974), while the salivary glands of the blowfly, *Calliphora vomitoria*, are devoid of innervation altogether (Oschman and Berridge 1970). Even though salivary gland innervation patterns vary within the insect class, certain neuroactive substances, such as serotonin and dopamine, appear to be favoured sialagogues. Either one or both amines have been found associated with the salivary glands of locusts, cockroaches, true bugs, moths, crickets and mosquitoes (Gifford *et al.*, 1991; Orchard *et al.*, 1992; Baines *et al.*, 1989; Tyrer *et al.*, 1984; Davis 1985; Orchard *et al.*, 1988; Robertson 1975; Hörner *et al.*, 1995; Novak *et al.*, 1995).

The biogenic amines serotonin and dopamine have been found throughout the nervous system of a wide variety of insects by immunohistochemical and chromatographic detection techniques (Evans 1980; Brown and Nestler 1985). Our understanding of their physiological roles is at an early stage, but studies of salivary gland preparations have furthered our knowledge of these roles, particularly those studies performed on blowfly and cockroach salivary glands.

The salivary glands of the cockroach are acinar and are innervated via a suboesophageal salivary nerve which contains 2 large axons and several smaller diameter ones (Whitehead 1973). Of the two large axons, one (salivary neurone 1; SN1) is dopaminergic while the phenotype of the other is unknown. The smaller diameter axons probably belong to a
serotonergic neurohaemal system and are found closer to the surface of the nerve (Davis 1987; Whitehead 1973). The glands also receive projections from the stomatogastric nervous system via small serotonergic neurohaemal axons which form neurohaemal areas (Davis 1985). Thus, cockroach salivary glands receive serotonergic and dopaminergic innervation. Serotonin increases the salivation rate in Periplaneta americana from basal levels (0 nl/min) to a maximum of 379±43 nl/min at a concentration of 10⁻⁵ M (Just and Walz, 1996). Since the saliva secreted in response to serotonin has a high protein content (Just and Walz, 1996), it is likely that serotonin acts on the central cells of the acini to induce an exocytosis of protein into the lumen of the duct. Analysis of the catecholamine content of cockroach salivary glands and suboesophageal salivary neurones (Gifford et al., 1991; Fry et al., 1974) confirms the presence of dopamine in the glands. Dopamine induces the salivation of water and ions in Periplaneta americana without affecting the protein content of the saliva (Just and Walz, 1996). Dopamine is capable of increasing the cyclic AMP levels in this tissue, which in turn is responsible for inducing salivary secretion (House and Ginsborg 1985; Grewe and Kebabian 1982). However, dopamine is also capable of changing the acinar membrane potential while cyclic AMP is not, thus leading to the hypothesis that dopamine binds to 2 classes of receptors: the first is positively linked to the enzyme adenylate cyclase, while the second is associated with the acinar membrane potential, and probably affects it via a Ca²⁺-mediated mechanism (House and Ginsborg 1985).

The salivary glands of the blowfly are not innervated, but are acted upon in a neurohormonal fashion by serotonin and possibly by several members of the FMRFamide family of peptides (Duve et al., 1992; Berridge and Patel 1968). Serotonin appears to act on two separate receptors in the salivary glands of Calliphora to induce salivation: the first (5-HT₁) mediates calcium elevation within the acini which is responsible for a chloride-dependent depolarizing response of the acinar cells (Prince and Berridge 1972). The other (5-HT₃) mediates fluid secretion via a cyclic AMP second messenger system (Berridge and Heslop 1980; Fain and Berridge 1979; Berridge 1970) which involves a hyperpolarizing response of the acinar cells (Prince and Berridge 1972). Three
Figure 1. Suboesophageal ganglion of *Locusta migratoria*. The two salivary neurons are illustrated (SN1 and SN2, each neuron is one member of a bilateral pair) which project to nerve 7b (NV7b) to the salivary gland. Black cell body is dorsal while the clear cell body is ventral.
FMRFamide related peptides recently sequenced from the thoracic ganglia of *Calliphora* have been shown to increase the rate of fluid secretion in the *Calliphora* salivary gland bioassay (Duve et al., 1992). The most potent of these peptides, CalliFMRFamide 1, is physiologically active at a concentration of 0.1-1 nM (Duve et al., 1992) which is comparable to the potency of serotonin that has a threshold of activity of approximately 1 nM (Berridge and Patel 1968). The physiological significance of having several substances capable of inducing salivation is still undetermined.

Little is known about the innervation of the salivary glands of other insects. For instance, the salivary glands and ducts of *Rhodnius prolixus* are covered in a dense plexus of serotonergic (Orchard et al., 1988) and FMRFamide-like immunoreactive fibres (Tsang and Orchard 1990) but further physiological studies have not been performed. An interesting finding is the positive serotonin-like immunoreactivity associated with the salivary glands of female *Aedes aegypti* (bloodfeeders) but its absence in males (non-bloodfeeders) (Novak et al., 1995), suggests a correlation between serotonin and bloodfeeding in this animal in a manner similar to that in *Rhodnius*. Axon swellings of serotonergic neurohaemal elements on nerves closely associated with the salivary glands of the Colorado potato beetle, *Leptinotarsa decemlineata*, may indicate a possible salivary gland target for serotonin (van Haeften and Schooneveld, 1993). However, no physiological or biochemical studies of any kind have been carried out to test this. Interestingly, the tubular glands of the adult *Manduca* are innervated via dopaminergic fibres which arise from the oesophageal nervous system (Robertson 1974, 1975) while the labial glands of the imago are not innervated and are specialised for silk production. The salivary glands of the cricket, *Gryllus bimaculatus*, are innervated via a salivary nerve from the SOG which contains a dopaminergic SN1 and a serotonergic SN2 (Horner et al., 1995). The morphology of these salivary neurons is very similar to that of the salivary neurons in *Periplaneta americana* and *Locusta migratoria*.

**Locust Salivary Glands**

The salivary glands of the locust, *Locusta migratoria*, are innervated via two
motoneurons (salivary neuron 1 and 2; SN1, SN2) whose somata are located within the SOG (Fig 1; Altman and Kien 1979). The SN1 are located on the dorsal surface of the ganglion in the anterior region. They have a characteristic morphology and project to the contralateral salivary nerve root, where they run to the salivary gland and branch over the acini. The terminals of the SN1 are intimately associated with the cells of the acini. Several nerve terminal release sites have been identified, but actual synapses have not been located (Peters et al., 1987). The somata of the SN2 are located on the ventral surface of the labial neuromere of the SOG and project ipsilaterally to the salivary nerve and then to the salivary gland (Altman and Kien, 1979) where their terminals are found associated with the cells of the acini. Both the SN1 and the SN2 have been found to stain positively with an anti-serotonin antiserum (Tyrer, et al., 1984). In addition, tyrosine hydroxylase-like immunoreactivity of the locust ventral nerve cord reveals the presence of this enzyme in the SN1 and in their terminals in the salivary glands (Orchard, et al., 1990). Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the pathway for the production of catecholamines and positive TH-like immunoreactivity implies the presence of catecholamines. Since insects appear to have no adrenaline and since noradrenaline levels are relatively low (Evans, 1980), TH is indicative of the presence of dopamine. Thus it appears that while the SN2 in Locusta contain serotonin, the SN1 may contain both serotonin and dopamine. Stimulation of both SN1 and SN2 is capable of increasing salivary secretions in the locust (Baines and Tyrer, 1989). It is likely that dopamine and serotonin are capable of activating the cells of the acini to induce secretion. The acini of the desert locust Schistocerca gregaria are composed primarily of two cell types: the zymogenic and parietal cells (Kendall 1969). The parietal cells are conical in shape with their apex pointing toward the center of the acini. They contain many mitochondria and are believed to accumulate raw material used for the production of salivary enzymes and proteins (Kendall 1969). The zymogenic cells are larger and are situated more to the interior of the acini. It is presumed that they complete the production of salivary enzymes which was initiated by the parietal cells (Kendall 1969). The granular, proteinaceous secretions are eventually secreted into the ducts.
It is probable that dopamine and serotonin bind to receptors on the surface of the zymogenic and parietal cells to induce salivation. However, little is known of the receptor types for these amines associated with the salivary glands. A preliminary study suggested the presence of dopaminergic receptors that may be similar to \( D_1 \) type receptors and that are coupled to the enzyme adenylate cyclase (Lafon-Cazal and Bockaert, 1985). Nothing is known of the serotonergic receptor types associated with this tissue. Long term recording from the salivary nerve of the locust reveals that the SN2 are active at 4-5 Hz when the animal is feeding, and are inactive in non-feeding animals (Schactner and Bräunig, 1995). The SN1 are also active during feeding at approximately 6-8 Hz and in the non-feeding animal they are periodically active at 5-8 Hz (Schactner and Bräunig, 1995). These differences in activity suggest that these neurons may perform slightly different functions.

Additional serotonergic innervation of the salivary gland arises from a neurohaemal network that is closely associated with the surface of the salivary nerve and that has its origins from somata which lie in the anterior region of the SOG (Bräunig 1987, 1988). This serotonergic system, or satellite nervous system (SNS), projects along the surface of the salivary nerve and terminates just short of the acini. Hence, additional serotonergic control of salivation may come from this neurohaemal source.

Locust salivary glands also receive projections from branches of the transverse nerves which arise from the prothoracic and mesothoracic posterior median nerves and which stain positively for FMRFamide-like immunoreactivity (Evans and Myers 1985; Fusé et al., 1996). Although the physiological roles of these peptides are yet to be assessed, an earlier study suggested that stimulation of the transverse nerve increased the amount of saliva produced, increased the activity of the SN1 and suppressed the activity of the SN2 (Baines and Tyrer, 1989). In addition to this, we know that AFIRFa and GQERNFLRFa (2 Locusta FMRFa-related peptides) are unable to alter cyclic AMP levels in the salivary glands when applied to whole gland preparations (Fusé et al., 1996).

**Objectives**

This study was undertaken in order to more fully investigate the aminergic innervation
of *Locusta* salivary glands. Questions have arisen concerning the colocalization of serotonin and dopamine in the SN1, which seems a curious occurrence in light of the serotonergic nature of the SN2 and the SNS, and in light of the different activity levels of the salivary neurons. This study also investigates the roles of serotonin and dopamine as true neurotransmitters in this system. In order to do this it must be shown that serotonin and dopamine fulfill the neurotransmitter criteria at the salivary glands. These criteria are the following: there must be a mechanism of removal of the substance from the synaptic cleft; the substance must be present within the presynaptic neuron; enzymes for the substance's synthesis must be present in the presynaptic neuron; the substance must be released by specific stimulation of the presynaptic neurone; the substance must have identical actions as the natural transmitter; and, the substance must have identical pharmacology to the natural transmitter. In addition, a preliminary study of the different elements that may be involved in the control or activation of the salivary neurons is also performed. Thus, the possibility of salivary neuron activation via other nerve roots on the SOG is examined, in addition to investigating the ability of putative neurotransmitters to activate SN1 or SN2. Finally, a comparative study between the innervation patterns of the salivary glands of *Locusta migratoria*, the cockroach *Periplaneta americana*, and the stick insect *Carausius morosus* is performed, not only as a necessary initial study for future physiological work, but also for this information to be used to examine the evolutionary and phylogenetic relationships of these animals.

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II. The aminergic control of locust (*Locusta migratoria*) salivary glands: evidence for dopaminergic and serotonergic innervation.

Abstract

Tyrosine hydroxylase-like immunoreactivity and serotonin-like immunoreactivity of the suboesophageal ganglion (SOG) of the locust, *Locusta migratoria*, reveal several pairs of cells. One pair of tyrosine hydroxylase-like positive cells (SN1) and one pair of serotonin-like positive cells (SN2) both project through nerve 7b to the salivary glands, where they branch extensively over the acini. Individual acini receive innervation from both SN1 and SN2. Co-localization of immunoreactivity was never observed. Neurons that stain positively for tyrosine hydroxylase-like immunoreactivity are considered to be catecholaminergic since tyrosine hydroxylase is the first and rate limiting enzyme in the pathway for the production of catecholamines. High-performance liquid chromatography of the salivary glands reveals the presence of dopamine and serotonin. Both dopamine and serotonin induce an elevation of cyclic AMP in salivary glands of adult male locusts in a dose-dependent manner. Thus it appears that cyclic AMP is a second messenger for the action of dopamine and serotonin on salivary glands. The presence of distinct dopamine and serotonin receptors on the glands was shown via the ability of the receptor antagonists, SCH-23390 and ketanserin to inhibit the elevation of cyclic AMP levels induced by dopamine and serotonin respectively.
Introduction

Dopamine and serotonin are biogenic amines found throughout the nervous system of a variety of insects (Evans, 1980; Brown and Nestler, 1985) where they are thought to perform roles as neurotransmitters and neurohormones (Collins and Miller, 1977; Trimmer 1985; Maddrell et al., 1971; Brown 1967; Orchard et al., 1988). However, in spite of their apparent importance, there is still relatively little information regarding their precise physiological functions. The discovery of identifiable dopaminergic and serotonergic neurons is an important step towards a description of the physiological relevance of these amines in the insect nervous system, in a manner parallel to that used so successfully for octopamine (see Evans 1980; Orchard 1982; Orchard and Lange, 1985). Means of identifying dopamine and serotonin have concentrated on the use of either radioenzymatic assays (Gifford et al., 1991) or immunohistochemical methods (Budnik and White, 1988a; Nässel 1988; Schäfer and Rehder, 1989; Orchard 1990; Orchard et al., 1992). Antibodies against either amines or enzymes in the pathway for the production of amines have been used effectively to identify aminergic neurons (Klemm et al., 1985; Konrad and Marsh, 1987; Budnik and White 1988a; Orchard et al., 1992). Localization of the enzyme tyrosine hydroxylase, which catalyzes the first and rate limiting step in the synthesis of catecholamines, has been used to identify catecholaminergic neurons. Tyrosine hydroxylase-like immunoreactive neurons correspond well with catecholamine-containing neurons in vertebrates (see Davis and Jang, 1988) and invertebrates (Budnik and White, 1988a; Orchard 1990; Orchard et al., 1992; Vieillemaringe et al., 1984) and so the localization of tyrosine hydroxylase appears to be a reliable method for identifying catecholaminergic neurons.

Insect salivary glands are useful preparations for the study of aminergic control mechanisms. Serotonin is a modulator controlling salivary gland secretions in the blowfly, Calliphora erythrocephala (Berridge 1970, 1972), whereas dopamine stimulates fluid secretion in the cockroach (Bowser-Riley and House, 1976). The salivary glands of Locusta migratoria are directly innervated from the suboesophageal ganglion via
bilaterally paired nerves, 7b, and have been shown to possess catecholaminergic terminals (Klemm 1972). Cobalt backfilling of nerve 7b reveals the presence of only two axons within this nerve (Altman and Kien, 1979), arising from two neurons, SN1 and SN2, whose somata are located in the suboesophageal ganglion. Both neurons were initially reported to be serotonergic (Tyrer et al., 1984); however, later studies revealed that SN1 stained positively for dopamine using Falk-Hillarp fluorescence, and radioenzymatic assays of this neuron showed the presence of dopamine but no significant amounts of serotonin (Gifford et al., 1991). More recently, tyrosine hydroxylase-like immunoreactive staining of the locust ventral nerve cord (Orchard et al., 1992) has shown that SN1 stains positively for tyrosine hydroxylase, confirming the probable catecholamine content of this neuron. Thus, there remains some confusion in the literature as to the identity of the amines present in these salivary neurons. It is possible that both SN1 and SN2 are serotonergic with the catecholamine, dopamine, colocalized in SN1, or that SN1 is dopaminergic and SN2 is serotonergic with no colocalization.

The aim of the present study was to investigate further the serotonergic and dopaminergic nature of SN1 and SN2 of the suboesophageal ganglion and their innervation of the salivary glands of Locusta migratoria. Concurrently, the effects of dopamine and serotonin on salivary gland cyclic AMP levels was also studied in order to provide some basis for the pharmacological classification of receptor types, since evidence has accumulated for the involvement of a cyclic AMP second messenger system in the control of insect salivary glands (Berridge 1970; Berridge and Heslop, 1981; Grewe and Kebabian, 1982).

Materials and Methods

Insects

Adult males of Locusta migratoria were taken 2 to 3 weeks post ecdysis from a crowded colony maintained at 30°C under a 12 hour light:12 hour dark regime. Insects were fed
daily on freshly grown wheat supplemented with bran.

**Immunohistochemistry**

Immunohistochemical studies were performed on isolated suboesophageal ganglia and salivary glands which were dissected under physiological saline (150 mM NaCl; 10 mM KCl; 4 mM CaCl₂; 2 mM MgCl₂; 4 mM NaHCO₃; 5 mM HEPES; pH 7.2; 90 mM sucrose; 5 mM trehalose). Tissues were fixed in 2% paraformaldehyde in Millonig's buffer for 1 hour, washed in phosphate-buffered saline (10 mM phosphate buffer, pH 7.2 containing 0.9% NaCl) for 2 hours and then incubated in 4% Triton X-100 in phosphate buffered saline for 1 hr. The tissues were then processed for either tyrosine hydroxylase-like or serotonin-like immunoreactivity following previously described techniques (Lange et al., 1988; Orchard, 1990; Orchard et al., 1992). Briefly, for tyrosine hydroxylase-like immunoreactivity, the tissues were incubated at 4°C in a mouse monoclonal antibody generated against tyrosine hydroxylase (Incstar Corp., Stillwater, MN) diluted 1:500 in phosphate buffered saline containing 2% bovine serum albumin, 2% normal goat serum and 0.4% Triton-X100. After 48 hours the tissues were washed in phosphate buffered saline for 6 hours prior to incubation in the secondary antibody for 18 h at 4°C. The secondary antibody was a goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA), labelled with fluorescein isothiocyanate diluted 1:200 in phosphate buffered saline containing 10% normal goat serum. Tissues were washed in phosphate buffered saline and subsequently cleared and mounted on depression slides in 5% n-propyl gallate in 80% glycerol (pH 7.3).

Serotonin-like immunoreactivity was investigated using similar techniques except that tissues were incubated in a 1:1000 dilution of a rabbit anti-serotonin antibody (Incstar Corp.) and the secondary antibody was a goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories) labelled with fluorescein isothiocyanate.

Double labelling, using both the tyrosine hydroxylase antibody and serotonin antibody, was also performed. In these preparations the two primary antibodies were applied together, whereas the secondary antibodies were applied sequentially. Goat anti-rabbit immunoglobulin G labelled with fluorescein isothiocyanate was used to detect serotonin-
like immunoreactivity and goat anti-mouse immunoglobulin G labelled with rhodamine was used to detect tyrosine hydroxylase-like immunoreactivity.

**Electrochemical detection of dopamine and serotonin**

Quantification of dopamine and serotonin in salivary glands was performed using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (see Orchard 1990; Lange et al., 1988). Salivary glands were dissected under physiological saline and placed in 110 µl of HPLC buffer consisting of 62.5 mM NaH₂PO₄, 1.5 mM sodium dodecyl sulphate, 1 µM EDTA, 15% methanol and 16.2% acetonitrile adjusted to pH 3.3 with perchloric acid. This mixture was sonicated, centrifuged at 8800 g, and filtered through a 0.22 µm filter before injection. Samples were injected onto a Brownlee ODS-Spheri 5 HPLC column (4.6 mm x 22 cm), and separated using the mobile phase (HPLC buffer) under a flow rate of 0.8 ml/min. Eluted compounds were detected electrochemically using an ESA model 5100 detection system coupled to a model 5010A dual coulometric detector (ESA Inc. Bedford, Mass.). The first detector, set at 0.05 V, acted as a screen while a guard cell inserted before the injection valve was set at 0.4 V to preoxidise possible contaminants in the mobile phase. The output of the second detector (set at 0.35 V) was recorded on a Spectra Physics 4270 Integrator (Spectra Physics, San Jose, Calif.) and dopamine and serotonin levels quantified using the external standard method. Samples were spiked with dopamine and serotonin to confirm the identity of the oxidizable substances and to check for losses.

**Cyclic AMP measurements**

Salivary glands were dissected under physiological saline and assayed for the effect of various agents on cyclic AMP levels. Tissues were incubated in physiological saline containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), along with various concentrations of dopamine or serotonin for 10 min. at room temperature. In experiments designed to investigate the effects of various receptor antagonists, the tissues were incubated in the antagonist and IBMX for 5 min. at room temperature prior to the addition
of dopamine or serotonin. At the end of the incubation period, the reaction was terminated by addition of 500 µl boiling 0.05 M sodium acetate buffer, pH 6.2, followed by 5 min boiling. The samples were stored at -20°C until further use. The samples were sonicated, centrifuged at 8800g for 10 min., and the supernatant removed for cyclic AMP determination while the pellet was dissolved in 50 µl 0.5 N sodium hydroxide for protein determination. Cyclic AMP levels were measured by radioimmunoassay (see Lange and Orchard, 1986a) using a commercially available kit (New England Nuclear). The protein content of the salivary glands was determined using the Bio-Rad protein assay based upon Bradford (1976) using gamma globulin as standard.

All chemicals were obtained from Sigma Chemical Co. (St Louis, Mo.) except for ketanserin and SCH-23390 which were obtained from Research Biochemicals Inc. (Natick, MA).

Results

Immunohistochemistry

Locust salivary glands are innervated from the suboesophageal ganglion via the bilaterally paired nerves 7b, the salivary nerves (Altman and Kien, 1979). Each nerve contains the axons of only 1 pair of motoneurons, the SN1 and SN2 (see Altman and Kien, 1979; Gifford et al., 1991). These two neurons have distinctive branching patterns with the cell body of SN1 located dorsally in the anterior region of the suboesophageal ganglion and having its axon run contralaterally then posteriorly along the lateral portion of the ganglion to exit via nerve 7b, whereas the cell body of SN2 is located ventrally and posteriorly and has its axon projecting ipsilaterally through the nerve 7B.

As previously shown (Orchard et al., 1992), immunohistochemistry reveals that the bilaterally paired SN1 are positively stained using the tyrosine hydroxylase antibody (Fig 1a) and each has a cell body diameter of approximately 80 µM. A single brightly stained axon traverses the suboesophageal ganglion to exit nerve 7b to the salivary gland (Fig
Figure 1: Wholemount preparations of tyrosine hydroxylase-like immunoreactive staining of suboesophageal ganglion and salivary glands of *Locusta*. (a) Suboesophageal ganglion showing cell bodies of the salivary neurons (SN1) and their contralaterally projecting axons (arrows). (b) Axon of SN1 (arrow) as it projects out of the suboesophageal ganglion through nerve 7b. (c) Axon of SN1 in nerve 7b as it branches (small arrow) from the main axon which follows the salivary duct (thick arrow). (d) Process and smaller branches among acini (arrows). (e) (f) Network of immunoreactive staining over the acini of the salivary glands (black arrows). Scale bar: (a),(d) 200 μm; (b),(c),(e),(f) 110 μm.
Figure 2: Wholemount preparations of serotonin-like immunoreactive staining of the suboesophageal ganglion and salivary glands of *Locusta*. (a) Suboesophageal ganglion showing cell bodies (arrow) of salivary neurons (SN2). (b) Axon of SN2 and a branch (thin arrows) running along the salivary duct (thick arrow). Shown here as a distinct process (white arrows). (c) Neurohaemal-like staining pattern (curved arrows) associated with nerve 7b. (d) Neurohaemal-like staining on nerve 7b (curved arrow), smaller processes leading onto the acini (white arrow) and the network staining pattern over the acini (black arrow). (e) Axonal projections leading onto the acini (thin arrow) and network of immunoreactivity (thick arrow). (f) Network of serotonin-like immunoreactivity over a cluster of salivary gland acini. Scale bar: (a) 200 μm; (b)-(f) 110 μm.
1a,b). Here the axon follows the salivary duct and thence branches over the gland (Fig 1c,d). Positively stained processes project to the salivary gland acini where they further branch over the acini forming a dense network (Fig 1e,f).

Serotonin-like immunohistochemistry reveals weaker staining of the bilaterally paired SN2. Their somata are located in the posterior median region of the ganglion closer to its ventral surface (Fig 2a). Each cell body is approximately 50 μm in diameter and projects an axon ipsilaterally to exit the ganglion via nerve 7b and on to the salivary gland (Fig 2b). Only one axon positively stained for serotonin-like immunohistochemistry was ever seen in each of nerve 7b. Serotonin-like staining of nerve 7b displayed some interesting features. In many preparations the nerve exhibited a profuse neurohaemal-like staining pattern with numerous branches covering the nerve (Fig 2c,d). Indeed, this pattern appeared to be initiated at the junction of nerve 7 and the suboesophageal ganglion, progress along the nerve to the salivary gland, and then extend over the acini. On occasion, however, the profuse neurohaemal-like pattern was not evident and in its place, there was a single, distinct axon within nerve 7b as it ran along the duct (Fig 2b). Small projections could be seen extending over the acini and spreading outwardly into a diffuse network over the acini (Fig 2d,e,f).

To further confirm the distinct staining of the SN1 and SN2, double labelling was employed. In the same preparations we were able to stain both axons and their branches onto the acini. The staining was always distinct, one axon and its branches positive for tyrosine hydroxylase-like immunoreactivity, the other axon and its branches positive for serotonin-like immunoreactivity (data not shown). Individual acini were observed receiving innervation from both axons. Co-localization was never observed.

The SN1, which stained positively for tyrosine hydroxylase-like immunoreactivity, exhibited no serotonin-like immunofluorescence in any preparation. Likewise, the SN2, which stained positively for serotonin-like immunoreactivity, exhibited no tyrosine hydroxylase-like immunofluorescence.

Electrochemical detection of dopamine and serotonin

Confirmation of the aminergic content of the locust salivary glands was obtained
Figure 3: HPLC trace of Locusta salivary glands showing (a) two peaks which co-migrate with dopamine and serotonin standards. (b) Same salivary gland extract spiked with dopamine (1.8 pmol) and serotonin (2.6 pmol).
using HPLC coupled to electrochemical detection. The salivary glands contain two oxidizable substances with the same retention times as dopamine and serotonin on HPLC (Fig 3a). When the same sample was spiked with dopamine and serotonin (Fig 3b), the two oxidizable peaks were increased in area and still remained as single peaks with no shoulders, thereby confirming their probable identities as dopamine and serotonin. Quantification of dopamine and serotonin indicate that the dopamine content of the gland is approximately 39.9 ± 7.1 pmol/mg of protein whereas the serotonin content is approximately 44.0 ± 5.4 pmol/mg of protein (mean ± standard error of 4 determinations).

**Cyclic AMP content**

Incubation of salivary glands with serotonin in the presence of IBMX resulted in a dose-dependent increase in cyclic AMP content. A sigmoid relationship between the log of serotonin concentration versus the increase in cyclic AMP content is clearly evident (Fig 4). The threshold for cyclic AMP elevation occurred at approximately 0.05 μM, with maximal elevation at 5 μM. A 50% maximum response was achieved at 0.1 μM serotonin.

Similarly, the effect of dopamine on cyclic AMP content was investigated. Dopamine caused a dose-dependent increase in cyclic AMP content (Fig 5). This relationship also appeared to follow a sigmoid curve when the increase in cyclic AMP was plotted against the log of dopamine concentration. The threshold for cyclic AMP elevation occurred at a dopamine concentration of 0.01 μM, whereas maximally elevated levels occurred at approximately 10 μM. A 50% maximum response was achieved with approximately 0.2 μM dopamine. Interestingly, the dopamine-induced cyclic AMP elevation appears to exhibit a plateau-like phase over the range of dopamine concentrations from 0.5 μM to 5 μM, although the standard errors are quite large at these points.

The ability of serotonin and dopamine receptor antagonists to inhibit the agonist-induced elevations of cyclic AMP was examined to further characterize the receptor types. The vertebrate serotonin receptor antagonist, ketanserin (5 μM), was able to partially, though significantly inhibit ( p<0.05, one way ANOVA performed on a Duncan's & Student Newman-Keuls test) the serotonin-induced elevation in cyclic AMP, but had no significant effect on dopamine-induced elevation in cyclic AMP (amines used at 0.5 μM)(Fig 6).
Figure 4: Increase in cyclic AMP content/mg protein of salivary glands of adult male *Locusta* following incubation for 10 min. in different concentrations of serotonin and 0.5 mM IBMX. Values are means ± S.E. (*n* > 5) and reflect increases in cyclic AMP (per mg protein) over IBMX control values.
Increase in cAMP (pmol/mg protein)

Serotonin (M) log scale

10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
Figure 5: Increase in cyclic AMP content/mg protein of salivary glands of adult male 
*Locusia* following incubation for 10 min. in different concentrations of 
dopamine and 0.5 mM IBMX. Values are means ± S.E. (n > 5) and reflect 
increases in cyclic AMP (per mg protein) over IBMX control values.
Figure 6: Effect of the serotonergic antagonist, ketanserin, on the cyclic AMP content/mg protein of salivary glands in the presence of 0.5 mM IBMX and either 0.5 μM dopamine or 0.5 μM serotonin. Ketanserin partially inhibits the effects of serotonin but has no effect on dopamine.
Figure 7: Effect of the dopaminergic antagonist, SCH-23390, on the cyclic AMP content /mg protein of salivary glands in the presence of 0.5 mM IBMX and either 0.5 μM dopamine or 0.5 μM serotonin. SCH-23390 inhibits the effects of dopamine but has no effect on serotonin.
Conversely, a specific vertebrate type D1 receptor antagonist, SCH-23390 at 5 μM was able to significantly inhibit (p<0.05, one way ANOVA performed on a Duncan’s & Student Newman-Keuls test) the elevation in cyclic AMP induced by 0.5 μM dopamine, but had no significant effect on the serotonin-induced elevation in cyclic AMP (Fig 7).

Discussion

The use of tyrosine hydroxylase-like immunohistochemistry to identify catecholaminergic neurons is a simple and reliable technique. First carried out in vertebrates (see Davis and Jang, 1988; Saland et al., 1988; Kitahama et al., 1989) this technique has been successfully performed on invertebrate nervous systems (Budnik and White, 1988a,b) although not without scepticism since the synthetic pathway for catecholamine production in invertebrates may differ from that in vertebrates. However, published literature has shown that known catecholaminergic neurons correspond well with tyrosine hydroxylase-like immunoreactive cells and reports on the use of this powerful technique in invertebrates (Flanagan 1984,1986; Budnik and White, 1988a; Nyhof-Young and Orchard, 1990; Orchard 1990; Orchard et al., 1992) lends support to its reliability. Serotonin-like immunohistochemistry has been reported in a number of insect species (see Nässel 1988, for review) and reports concerning its high specificity (Steinbusch et al., 1983; Schipper and Tilders, 1983; Tandler et al., 1986) leave little doubt as to the reliability of this procedure. Immunohistochemistry has revealed a single serotonin-like immunoreactive axon within nerve 7b, as well as a single tyrosine hydroxylase-like immunoreactive axon. Double labelling confirms the distinct staining of individual axons. These results leave little doubt that neuron SN1 stains only for tyrosine hydroxylase-like immunoreactivity while SN2 stains only for serotonin-like immunoreactivity. It was Tyrer et al. (1984) who first advanced the claim that locust salivary glands are innervated from two separate serotonergic axons originating in the suboesophageal ganglion, but the earlier report concerning the presence of catecholaminergic terminals on the glands (Klemm 1972)
prompted further investigation of this system. Radioenzymatic assays positively identified the presence of dopamine in SN1 and serotonin in SN2 (Gifford et al., 1991) and Falk-Hillarp fluorescence of these neurons gave positive results for dopamine in SN1 only (Gifford et al., 1991). The Falk-Hillarp technique is not the ideal technique for identifying amines due to its low sensitivity. Similarly, radioenzymatic assays may not detect low levels of amines in neurons. Thus there was still the possibility of colocalization of dopamine and serotonin in SN1 and SN2. Certainly the colocalization of amines has been observed in vertebrate neurons which stained positively for tyrosine hydroxylase-like immunohistochemistry and serotonin-like immunoreactivity (Saland et al., 1988) and as well there have been reports concerning the coexistence of GABA and serotonin (Belin et al., 1983) and for GABA and dopamine (Gall et al., 1987; Vuillez et al., 1987) in single vertebrate neurons. However, using the sensitive techniques of immunohistochemistry, we have found no evidence for colocalization within SN1 or SN2 for catecholamines and serotonin. HPLC determination of the salivary glands confirmed that dopamine and serotonin are associated with the gland, and the most parsimonious explanation is that dopamine is delivered via SN1 and serotonin via SN2. We do not know why SN1 was earlier identified as also being serotonergic, but it has been suggested as a possibility that the serotonin antibody may have cross-reacted with the dopamine or its precursors in these neurons (see Gifford et al., 1991). It certainly seems unlikely from the present data and that of Gifford et al., (1991) that SN1 is serotonergic. That SN1 and SN2 differ in the transmitter they employ is also suggested by the difference in their physiology. Baines et al., (1989) found that the spike frequency in SN2 is about 20 times that of SN1 in locusts which are not feeding. While spike frequency increases in both neurons during feeding, the increase in SN1 is relatively greater than that in SN2. Furthermore, stimulation of the transverse nerve increases the firing rate of SN1 but suppresses the activity of SN2 (Baines and Tyrer, 1989). These differences suggest a difference in physiological functions for SN1 and SN2 on salivary gland, and reiterates the likely use of different transmitters.

The present results suggest that adenylate cyclase plays a role in the signal transduction
mechanism for dopamine and serotonin, and dose-response curves suggest that cyclic AMP may be the second messenger which mediates the effects of dopamine and serotonin on fluid secretion rates, although this has yet to be investigated. In the cockroach *Nauphoeta cinerea*, the neurotransmitter, dopamine, is associated with the salivary gland (Kapoor *et al.*, 1983), stimulates the production of cyclic AMP (Grewe and Kebabian, 1982) and increases the rate of fluid secretion in isolated preparations (Evans and Green, 1990).

Serotonin seems to work via a similar mechanism in the blowfly, *C. erythrocephala* where it has been found to have two effects: the first is to elevate cyclic AMP levels and the second is to increase fluid secretion rates (Berridge 1970). Studies carried out on locust salivary glands have indicated that both dopamine and serotonin increase fluid secretion from whole gland preparations (Baines and Tyrer 1989) and preliminary pharmacological classification of dopamine receptors on locust salivary gland membrane preparations shows that dopamine receptors are linked to adenylate cyclase (Lafon-Cazal and Bockaert, 1984).

Initial classification on whole salivary glands suggests that dopamine and serotonin bind to separate receptor types which are both linked to the enzyme, adenylate cyclase. The vertebrate dopamine receptor antagonist, SCH-23390 blocks the elevation of cyclic AMP induced by dopamine while having no significant effect on the serotonin-induced increase in cyclic AMP levels, whereas, the serotonin receptor antagonist ketanserin, partially blocks the serotonin-induced elevation of cyclic AMP and has no effect on dopamine-induced elevation of cyclic AMP. Partial blocking of the serotonin-induced cyclic AMP levels emphasizes that these receptor antagonists are defined in vertebrate systems and may not be as effective in insects.

In conclusion, we have shown that the salivary glands of *Locusta migratoria* are innervated by a single catecholaminergic neuron and by a single serotonergic neuron arising from the suboesophageal ganglion. The biogenic amines, dopamine and serotonin, are intimately associated with the salivary glands where they have been shown to elevate cyclic AMP levels in a dose-dependent manner. Furthermore, these amines appear to bind to different receptors, both of which are probably linked to adenylate cyclase. Further investigations into the receptor types of this tissue are warranted since these salivary
glands appear to be a suitable preparation with which to classify the molecular structure of dopamine and serotonin receptors in *Locusta migratoria*. In addition, the description of identified catecholaminergic and serotonergic neurons along with their target sites make this an ideal preparation for studying the neurobiology of aminergic neurons.

**References**


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III. Characterization of dopamine and serotonin receptors on the salivary glands of the locust, *Locusta migratoria*

**Abstract**

The salivary glands of the locust, *Locusta migratoria*, are innervated by two pairs of neurons, one of which, the SN1, contains dopamine, while the other, the SN2, contains serotonin. The pharmacological properties of dopamine and serotonin receptors associated with the salivary glands of *Locusta migratoria* were examined. These receptors appear to be linked to adenylate cyclase, and, when activated by dopamine or serotonin, induce increases in cyclic AMP levels in the glands. The increases in cyclic AMP are mimicked by electrical stimulation of the axons of the SN1 and SN2 which presumably release dopamine and serotonin. A number of aminergic agonists were tested for their ability to induce increases in cyclic AMP levels in the salivary glands. Of these compounds, only epinephrine and synephrine were capable of significantly elevating cyclic AMP levels, while octopamine and norepinephrine were without effect when tested at 10 μM. Additivity studies suggest that the receptors for serotonin and dopamine are distinct. The rank order of potency (based upon IC$_{50}$ values) of serotonergic antagonists of spiperone > cyproheptadine > methysergide > mianserin > ketanserin suggests the presence of receptors pharmacologically similar to vertebrate 5-HT$_2$ receptors. IC$_{50}$ values of the dopaminergic antagonists, with a rank order of potency of SCH-23390 > (±)-butaclamol > flupenthixol, suggest the presence of receptors similar to vertebrate D$_1$ receptors. The neurally-evoked increases in cyclic AMP are sensitive to receptor antagonists. Thus, SCH-23390, a dopamine receptor antagonist, and spiperone, a serotonin receptor antagonist, are each capable of partially inhibiting the neurally-induced increase in cyclic AMP.
Introduction

The biogenic amines, dopamine and serotonin are present throughout the nervous system of insects (Evans 1980; Brown and Nestler, 1985), where they appear to play important roles as neurohormones, neuromodulators or neurotransmitters (Baines et al., 1990; Trimmer, 1985; Orchard et al., 1988). Dopamine is thought to mediate, at least in part, fluid secretion from cockroach salivary glands (Bowser-Riley and House, 1976; Smith and House, 1977), whereas serotonin has been implicated in a variety of roles including fluid secretion from blowfly salivary glands (Berridge, 1970), modulation of muscle activity in the cricket (Baines and Downer, 1991), and cuticular plasticization in the blood-sucking bug, Rhodnius prolixus (Barrett and Orchard, 1990). While it may be argued that there is still relatively little known regarding the overall physiological actions of dopamine and serotonin in insects, it seems apparent that many of the dopaminergic and serotonergic receptors are linked to adenylate cyclase (Berridge and Heslop, 1981; Grewe and Kebabian, 1982; Barrett and Orchard, 1990; Baines and Downer, 1991). This has been shown to be the case in several tissue types, but most notably in salivary glands (Heslop and Berridge, 1980; Grewe and Kebabian, 1982; Ali et al., 1993). Serotonin induces dose-dependent increases in cyclic AMP levels in the salivary glands of Calliphora (Heslop and Berridge, 1980), while cockroach salivary glands are responsive to dopamine, which induces salivary secretions and increases intracellular cyclic AMP levels (House and Ginsborg, 1979; Grewe and Kebabian, 1982).

Both dopamine and serotonin increase fluid secretion rates in isolated preparations of locust salivary glands (Baines and Tyrer, 1989). The salivary glands of the locust are directly innervated from the suboesophageal ganglion via the bilaterally paired nerves 7b. Cobalt backfilling of nerve 7b reveals the presence of two neurons, SN1 and SN2, whose somata are located in the suboesophageal ganglion (Altman and Kien, 1979). Thus, this preparation provides us with a unique opportunity for studying neuronal innervation of salivary glands since it is relatively simple and well defined. In addition, the salivary nerve can be easily located and stimulated, and the different diameters of the axons within nerve 7b (Baines et al., 1989) allow for the differential stimulation of SN1 and SN2.
The most recent investigations into the neurochemistry of SN1 and SN2 indicate the presence of dopamine in SN1 and serotonin in SN2 (Gifford et al., 1991; Ali et al., 1993). These studies suggested the presence of aminergic receptors associated with locust salivary glands, and logically, the presence of transduction mechanisms to link the neurotransmitters with their ultimate effects. Typically, vertebrate dopamine receptors fall within two main classes; a D₁ receptor that is positively linked to adenylate cyclase, and a D₂ receptor which may be negatively linked to adenylate cyclase (Seeman, 1981). In addition, recent evidence reveals that the D₂ receptor may also be linked to the IP₃ second messenger system (Todd et al., 1992). Vertebrate serotonin receptors have been classified under three main headings, 5-HT₁, 5-HT₂ and 5-HT₃ based primarily upon radioligand binding studies (see Peroutka, 1988 for review).

We have previously demonstrated that dopamine and serotonin induce dose-dependent elevations of cyclic AMP in the salivary glands of Locusta migratoria (Ali et al., 1993). In the present study, we investigate the pharmacological properties of the receptors that are coupled to adenylate cyclase, and which mediate the effects of dopamine and serotonin on locust salivary glands. In addition, we have examined the effects of neurally-evoked neurotransmitter release from the terminals of SN1 and SN2, on the cyclic AMP content of the salivary glands.

Materials and Methods

Insects

Adult males of Locusta migratoria were taken 6 to 10 days post ecdysis from a crowded colony maintained at 30°C under a 12 hour light:12 hour dark regime. Insects were fed daily on freshly grown wheat supplemented with bran.

Cyclic AMP measurements

Salivary glands were dissected under physiological saline (150 mM NaCl; 10 mM KCl;
4 mM CaCl₂; 2 mM MgCl₂; 4 mM NaHCO₃; 5 mM HEPES, pH 7.2; 90 mM sucrose; 5 mM trehalose) and assayed for the effects of various reagents on cyclic AMP levels. In order to examine the ability of aminergic agonists to elevate cyclic AMP levels, salivary glands were incubated in 0.5 mM isobutyl methylxanthine (IBMX) and 10 µM of the appropriate receptor agonist for 10 min at room temperature. In experiments designed to determine the effects of receptor antagonists, salivary glands were incubated in the antagonist and IBMX for 5 min at room temperature prior to the addition of dopamine or serotonin. At the end of the incubation period the reaction was terminated by addition of 500 µl of boiling 0.05 M sodium acetate buffer, pH 6.2, followed by 5 min boiling. The samples were sonicated, centrifuged at 8800 g for 10 min., and the supernatant removed for cyclic AMP determination while the pellet was dissolved in 50 µl 0.5 N NaOH for protein determination. Cyclic AMP levels were measured by radioimmunoassay (Lange and Orchard, 1986) using a commercially available kit (New England Nuclear, Lachine, Quebec, Canada). The protein content of the salivary glands was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) based upon Bradford (1976) using gamma globulin as standard.

**Neurophysiology**

Salivary glands, with the salivary duct and nerve 7b still attached, were placed in a saline pool (200 µl) that contained 0.5 mM IBMX. Nerve 7b, including the salivary duct, was gently drawn into a suction electrode and stimulated, while a second suction electrode was applied distally to record the effects of stimulation. Tissues were stimulated at 15 Hz for 5 min with 100 µsec square pulses. The effect of receptor antagonists was tested on stimulated preparations by incubating tissues in saline baths containing 0.5 mM IBMX and the antagonist for 5 min at room temperature prior to stimulation. Upon completion of stimulation, the preparation and saline pool was added to 500 µl of boiling 0.05 M sodium acetate buffer pH 6.2, followed by 5 min of boiling. Cyclic AMP and protein determinations were carried out as described above.
Chemicals

The following drugs were obtained from Research Biochemical Inc. (Natick, MA, USA): (±)SKF-82958, (-)-TNPA, (±)SKF-38393, (±)-PPHT, (+)-SCH-23390, (±)-butaclamol, flupenthixol, chlorpromazine, haloperidol, (±)-sulpiride, spiperone, cyproheptadine, mianserin, methysergide, ketanserin, MDL 72222, propranolol, 2-methylserotonin, α-methylserotonin, (±)-DOI-hydrochloride, CGS-12066B, m-CPP, (±)-8-OH-DPA. Dopamine, serotonin, (+)-epinephrine, (-)-norepinephrine, DL-octopamine, DL-synephrine and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Time course for the effects of dopamine, serotonin and IBMX on cyclic AMP levels

A time course for the effects of various agents on cyclic AMP content is shown in Figs 1 and 2. Salivary glands incubated in saline or 0.5 mM IBMX alone experienced a steady rise in cyclic AMP content over a 15 min period to maximum values of 25.3 ± 2.8 or 53.9 ± 5.8 pmol/mg protein respectively. Maximum elevations of cyclic AMP were achieved within 5 to 7 min when tissues were incubated in 0.5 μM dopamine and IBMX (Fig 1) or serotonin and IBMX (Fig 2), and remained relatively unchanged for the remainder of the incubation period. A 10 min incubation period was chosen for all subsequent experiments since this would allow the elevation of cyclic AMP to be complete. 0.5 μM Serotonin alone, induced a gradual accumulation of cyclic AMP in salivary glands over the first 10 min of incubation, achieving a maximum value of 83.2 ± 13.8 pmol/mg protein (Fig 2). Thus serotonin alone was more effective than IBMX in elevating cAMP levels. Glands incubated in 0.5 μM dopamine alone experienced only a small accumulation of cyclic AMP which was only a little different from the accumulation obtained in saline alone, and consequently dopamine was less effective than IBMX.

Additivity studies

Salivary glands incubated in 50 μM dopamine and 0.5 mM IBMX experienced an
Figure 1: Time course for the effects of saline (■), 0.5 mM IBMX (●), 0.5 μM dopamine (△), and 0.5 mM IBMX plus 0.5 μM dopamine (◇), upon the cyclic AMP content of salivary glands. Values represent mean ± S.E. (n>5).
Figure 2: Time course for the effects of saline (■), 0.5 mM IBMX (●), 0.5 μM serotonin (▲), and 0.5 mM IBMX plus 0.5 μM serotonin (▼), upon the cyclic AMP content of salivary glands. Values represent mean ± S.E. (n>5).
Figure 3: Cyclic AMP content of salivary glands, following incubation for 10 min in 50 μM of either dopamine or serotonin. IBMX (0.5 mM) included in all preparations. Values are mean ± S.E. (n>5).
accumulation of cyclic AMP levels of 166.1 ± 46 pmol/mg protein, whereas salivary glands incubated in IBMX and 50 μM serotonin experienced an accumulation of 212.7 ± 39.6 pmol/mg protein (Fig 3). Salivary glands incubated in 50 μM dopamine and 50 μM serotonin in the presence of 0.5 mM IBMX accumulated a cyclic AMP content of 365.2 ± 26.4 pmol/mg protein (Fig 3). The additive effect of these amines suggests the presence of discrete receptors for dopamine and serotonin.

**Effect of various amines**

The biogenic amines, norepinephrine and octopamine, did not significantly elevate cyclic AMP levels (Mann-Whitney test with Bonferroni correction) in salivary glands when tested at 10 μM (Fig 4), whereas, synephrine, the N-methylated derivative of octopamine, induced a statistical elevation of cyclic AMP (approximately 37.1% and 53.4% that of serotonin and dopamine respectively; Fig 4). Epinephrine was also capable of inducing elevations of cyclic AMP levels (18.3% and 26.3% that of serotonin and dopamine). The responses to serotonin and dopamine were greater than any of the other amines, and at 10 μM, induced maximally elevated cyclic AMP levels (Ali *et al.*, 1993).

**Effect of dopaminergic agonists**

The effects of dopaminergic agonists were tested on the salivary glands. The vertebrate D₁ receptor agonist, SKF-82958, was the only compound capable of inducing a statistically significant elevation of cyclic AMP levels in the tissue (Table 1) (Mann-Whitney test with Bonferroni correction). The remaining compounds, PPHT, TNPA and the other D₁ agonist, SKF-38393, were ineffective in inducing significant changes from control levels of cyclic AMP.

**Effect of dopaminergic antagonists**

The effectiveness of several vertebrate dopaminergic antagonists on the dopamine-induced increase in cyclic AMP levels of the salivary glands is reported in Table 2. SCH-23390, (±)-butaclamol and flupenthixol inhibited the dopamine-induced elevation of cyclic AMP. SCH-23390, a potent mammalian D₁ receptor antagonist inhibited the response to
Figure 4: Cyclic AMP content of salivary glands following incubation for 10 min in 10 μM amine. IBMX (0.5 mM) included in all incubations. Values represent mean ± S.E. (n=4-10).
Table 1: Action of dopaminergic agonists on cyclic AMP content of salivary glands of adult male *Locusta migratoria*. Cyclic AMP values are expressed as mean ± S.E. (n>5). Drugs tested at 10 μM in the presence of 0.5 mM IBMX.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Cyclic AMP (pmol/mg protein)</th>
<th>Percentage response relative to 10 μM dopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX</td>
<td>26.2 ± 2.3</td>
<td>0</td>
</tr>
<tr>
<td>Dopamine</td>
<td>99.8 ± 15.9*</td>
<td>100</td>
</tr>
<tr>
<td>SKF-82958</td>
<td>47.9 ± 6.2*</td>
<td>29.6</td>
</tr>
<tr>
<td>PPHT</td>
<td>29.4 ± 5.6</td>
<td>4.4</td>
</tr>
<tr>
<td>TNPA</td>
<td>27.5 ± 3.8</td>
<td>1.8</td>
</tr>
<tr>
<td>SKF-38393</td>
<td>23.2 ± 2.7</td>
<td>-4</td>
</tr>
</tbody>
</table>

* Values significantly different from controls
0.5 μM dopamine by 100% when tested at a concentration of 5 μM, and had the lowest IC₅₀ value of 0.25 μM, while butaclamol and flupenthixol had greater IC₅₀ values of 1.9 μM and 3.9 μM respectively. Chlorpromazine, haloperidol, spiperone and sulpiride had no effect on the dopamine-induced response. The rank order of potency of these drugs was SCH-23390 > butaclamol > flupenthixol.

**Effect of serotonergic agonists**

Serotonergic receptor agonists were tested for their ability to induce cyclic AMP elevations in the salivary glands. Agonists defined for vertebrate 5-HT₂ receptors induced a significant increase in cyclic AMP levels when compared to control values (Mann-Whitney test with Bonferroni correction) (Table 3). The most potent 5-HT₂ agonist, α-methylserotonin, induced a response approximately 39% the level of the serotonin-induced elevation. DOI-hydrochloride induced the second most potent response at 22% that of the serotonin control. Of the remaining agonists, only 2-methylserotonin, a 5-HT₁ agonist, induced a statistically significant increase in cyclic AMP levels reaching 47% that of the serotonin-induced elevation.

**Effect of serotonergic antagonists**

The antagonist with the greatest inhibitory effect on the serotonin-induced response was spiperone, which had the lowest IC₅₀ value of 4.4 μM (Table 4). Cyproheptadine, methysergide, mianserin and ketanserin were also capable of inhibiting the serotonin-induced elevation of cyclic AMP levels when tested at 10 μM. No drug tested was able to completely inhibit the serotonin-induced elevation of cyclic AMP. The 5-HT₃ antagonist, MDL-72222, and the 5-HT₁ antagonist, propranolol, had no inhibitory effect on the serotonin-induced response (Table 4). The rank order of potency for inhibition was spiperone > cyproheptadine > mianserin > methysergide > ketanserin.

**Stimulation of nerve 7b**

Extracellular stimulation of nerve 7b at 15 Hz for 5 min resulted in a significant increase in cyclic AMP levels in salivary glands (Fig 5), with cyclic AMP levels reaching increases
Table 2: Action of dopaminergic antagonists on dopamine-stimulated accumulation of cyclic AMP in salivary glands. Changes from basal level are expressed as mean ± S.E. (n>5). Drugs tested at 10 μM in the absence (change from basal level), or presence, of 0.5 μM dopamine. 0.5 mM IBMX included in all incubations. IC₅₀ represents the concentration of antagonist required to inhibit the effects of 0.5 μM dopamine by 50%. Basal level, 35.3 ± 3.5 pmol/mg protein.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Change from basal level (pmol/mg protein)</th>
<th>Percentage inhibition of 0.5 μM dopamine</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH-23390</td>
<td>5.9 ± 4.6</td>
<td>100</td>
<td>0.25</td>
</tr>
<tr>
<td>(+)-Butaclamoi</td>
<td>4.5 ± 4.4</td>
<td>80.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Flupenthixol</td>
<td>0.2 ± 1.9</td>
<td>50</td>
<td>3.9</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>3.7 ± 3.6</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>-5.0 ± 0.6</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Spiperone</td>
<td>4.6 ± 1.3</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>0.0 ± 2.9</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3: Action of serotonergic agonists on cyclic AMP levels in salivary glands of adult male *Locusta migratoria*. Cyclic AMP values are expressed as mean ± S.E. (n>5). Drugs tested at 10 μM in the presence of 0.5 mM IBMX.
Cyclic AMP (pmol/mg protein) relative to 10 µM serotonin

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cyclic AMP (pmol/mg protein)</th>
<th>Percentage response relative to 10 µM serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX</td>
<td>30.4 ± 2.4</td>
<td>0</td>
</tr>
<tr>
<td>Serotonin</td>
<td>155.4 ± 13.5*</td>
<td>100</td>
</tr>
<tr>
<td>2-methylserotonin</td>
<td>89.5 ± 11.6*</td>
<td>47.3</td>
</tr>
<tr>
<td>α-methylserotonin</td>
<td>78.9 ± 18.0*</td>
<td>38.7</td>
</tr>
<tr>
<td>DOI-hydrochloride</td>
<td>57.6 ± 5.2*</td>
<td>21.7</td>
</tr>
<tr>
<td>CGS-12066B</td>
<td>53.4 ± 5.3*</td>
<td>18.3</td>
</tr>
<tr>
<td>m-CPP</td>
<td>46.2 ± 15.7</td>
<td>12.6</td>
</tr>
<tr>
<td>8-OH-DPAT HBr</td>
<td>33.4 ± 3.5</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Values significantly different from controls.
Table 4: Action of serotonergic antagonists on serotonin-stimulated accumulation of cyclic AMP in salivary glands of adult male *Locusta migratoria*. Changes from basal level are expressed as mean ± S.E. (n>5). Drugs tested at 10 μM in the absence (change from basal level), or presence, of 0.5 μM serotonin. 0.5 mM IBMX included in all incubations. IC<sub>50</sub> represents the concentration of antagonist required to inhibit the effects of 0.5 μM serotonin by 50%. Basal level, 37.1 ± 3.7 pmol/mg protein.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Change from basal level (pmol/mg protein)</th>
<th>Percentage inhibition of 0.5 µM serotonin</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiperone</td>
<td>4.6 ± 1.3</td>
<td>64.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Cyproheptadine</td>
<td>7.3 ± 3.0</td>
<td>72.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Mianserin</td>
<td>-5.3 ± 3.5</td>
<td>56.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Methysergide</td>
<td>13.8 ± 4.0</td>
<td>64.4</td>
<td>8.4</td>
</tr>
<tr>
<td>Ketanserin</td>
<td>-4.7 ± 4.1</td>
<td>45.4</td>
<td>16</td>
</tr>
<tr>
<td>MDL 72222</td>
<td>-0.5 ± 6.4</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Propranolol</td>
<td>-6.7 ± 2.4</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
of 57.2 ± 9.9 pmol/mg protein. Salivary glands preincubated in 10 μM SCH-23390 achieved an increase in cyclic AMP of only 24.3 ± 6.5 pmol/mg protein, whereas glands preincubated in 10 μM spiperone accumulated cyclic AMP levels of only 28.6 ± 6.6 pmol/mg protein. The cyclic AMP accumulation in salivary glands that had been preincubated in both 10 μM SCH-23390 and 10 μM spiperone was 13.6 ± 3.2 pmol/mg protein.

Discussion

Additivity studies, combined with the ability of receptor antagonists to block the neurally-evoked increase in cyclic AMP, strongly suggest that the dopamine and serotonin receptors on the salivary glands of Locusta migratoria are separate and distinct entities. The salivary glands of the locust consist of clusters of acini that lie on both sides of the ventral thorax. The acini of the desert locust Schistocerca gregaria are composed of six different cell types of which the zymogenic and parietal cells are the most abundant (Kendall, 1969). These two main cell types also contain structures that appear to be actively involved in the production of salivary gland secretions (Kendall, 1969). It has been speculated that the parietal cells process raw materials and pass them on to the zymogenic cells which in turn produce granular secretions that are eventually expelled into the duct (Kendall, 1969). Attempts at studying the physiological response of the salivary glands have shown that secretory rates of this tissue are increased in the presence of dopamine and serotonin (Baines and Tyrer, 1989). Additionally, we had found that dopamine and serotonin induce dose-dependent increases in cyclic AMP levels of the glands of Locusta migratoria (Ali et al., 1993). Thus, it seems likely that dopamine and serotonin act on the zymogenic and parietal cells to induce salivary secretions, and that cyclic AMP may be an intermediate in this response. The location of these receptor sites is not known; however, we speculate that the adenylate cyclase-linked dopamine and serotonin receptors are concentrated on separate cell types. Since serotonin is able to induce an elevation of cyclic AMP in the absence of a phosphodiesterase inhibitor, whereas dopamine is not, we are led to believe that the activity of the phosphodiesterase is
Figure 5: The effects of neural activity upon the cyclic AMP content of the salivary glands. All values determined after 5 min stimulation following 5 min preincubation in 0.5 mM IBMX (stimulated tissues) and 10 µM antagonist. Increase in cyclic AMP content represents the increase from control tissues, which were concurrently run, non-stimulated salivary glands. Basal level, 35.7 ± 0.7 pmol/mg protein. Stim: stimulated; spip: spiperone; SCH: SCH-23390. All values represent mean ± S.E. (n=9-12).
low at the sites of serotonin action and high at the sites of dopamine action. Such a scenario might easily be explained if these amines acted on separate cells in which the phosphodiesterase activity was inherently different. That these receptor sites are similar to vertebrate D₁ and vertebrate 5-HT₂ receptors, is suggested by the profile of activity of receptor antagonists and agonists.

Dopamine receptors linked to adenylate cyclase have been reported to be present on cockroach salivary glands, brain and corpus cardiacum (Grewe and Kebabian, 1982; Orr et al., 1987; Gole et al., 1987), as well as on the salivary glands of the locust (Lafon-Cazal and Bockaert, 1984). Adenylate cyclase-coupled serotonin receptors were reported to be present on the cockroach corpus cardiacum (Gole et al., 1987), the cricket mandibular closer muscles (Baines and Downer, 1991), the epidermis of Rhodnius prolixus (Barrett and Orchard, 1990) and on Calliphora salivary glands (Heslop and Berridge, 1980).

In this report, we suggest that the dopamine receptors on Locusta salivary glands are pharmacologically similar to vertebrate D₁ receptors. Vertebrate D₁ receptors are positively linked to adenylate cyclase, and, upon activation, initiate a series of events that lead to the elevation of cyclic AMP. We have previously shown that dopamine induces dose-dependent increases in cyclic AMP levels (Ali et al., 1993), and here we show that certain dopaminergic receptor antagonists are capable of blocking the dopamine-induced elevation of cyclic AMP. The rank order of potency of the dopaminergic antagonists, SCH-23390 > butaclamol > flupenthixol > chlorpromazine (ineffective), suggests this receptor has a tendency to be similar to vertebrate D₁ receptors. These findings are in agreement with those of Lafon-Cazal and Bockaert (1984) who investigated the effects of dopamine and dopaminergic antagonists on the activity of adenylate cyclase in the salivary glands of the locust, and found evidence to suggest the presence of a D₁-like receptor.

The most effective antagonist, SCH-23390, is a potent and specific mammalian type D₁ receptor antagonist, while butaclamol and flupenthixol, also vertebrate D₁ antagonists, are less effective (Table 3). The lack of effectiveness of the D₂ antagonists, spiperone, sulpiride and haloperidol suggests the absence of D₂-like receptors. Vertebrate D₂ receptors may be negatively coupled to adenylate cyclase (Seeman, 1981) and, when activated, are
likely to induce decreases in cyclic AMP levels. Thus, inhibition of activated \( D_2 \) receptors should lead to elevated cyclic AMP levels. Using this criteria, we found no evidence for the presence of \( D_2 \)-like receptors, although these results are not conclusive and we have not ruled out the possibility of the presence of receptors linked to other second messenger systems.

The dopamine receptors in this preparation shows similarities with dopamine receptors on cockroach salivary glands. The profile for dopamine receptor antagonists on the secretory response of cockroach salivary glands, namely, chlorpromazine > SCH-23390 > haloperidol >> metoclopramide, suggests this receptor is similar to the vertebrate \( D_1 \) receptor (Evans and Green, 1990a). Evans and Green (1990b) also found that the dopamine receptor mediating the hyperpolarization response of cockroach salivary gland acini is similar to a \( D_1 \) receptor, even though cyclic AMP does not play a role in the electrical response of the acini (Gray et al., 1984). Cockroach brain dopamine receptors appear to be less distinct than those on locust salivary glands since they display properties similar to both vertebrate \( D_1 \) and \( D_2 \) receptors (Orr et al., 1987). In that preparation, LY-171555, a highly selective \( D_2 \) agonist elevated cyclic AMP levels. Additionally, both \( D_1 \) and \( D_2 \) antagonists significantly inhibited cyclic AMP production (Orr et al., 1987). Dopamine receptors on the cockroach corpus cardiacum, however, were selectively blocked by vertebrate \( D_1 \) antagonists, including cis-flupenthixol and (±)-butaclamol (Gole et al., 1987), and as such, were considered to be similar to vertebrate \( D_1 \) receptors.

Dopaminergic receptor agonists were relatively ineffective on locust salivary glands, since only the vertebrate \( D_1 \) agonist, SKF-82958, was able to partially elevate cyclic AMP levels. In some insect preparations, vertebrate receptor agonists have not been very potent in their ability to mimic the effects of the naturally occurring amine (Baines and Downer, 1991; Gole et al., 1987). This remains a curious characteristic of insect aminergic receptors. It is possible that the agonist binding sites are not only different from those of the natural amine, but are also more distantly related from their vertebrate counterpart.

The pharmacological profile of serotonergic antagonists and agonists suggests the presence of serotonin receptors similar to vertebrate 5-HT\(_2\) receptors. Vertebrate 5-HT\(_2\)
receptors tend to be linked to the phosphatidylinositol second messenger system (Peroutka, 1988), although, many insect serotonergic receptors, classified as 5-HT₂, are involved in the production of cyclic AMP (Berridge and Heslop, 1981; Gole et al., 1987; Barrett and Orchard, 1990; Baines and Downer, 1991). Serotonin elevates both cyclic AMP and IP₃ levels in Calliphora salivary glands, via receptors classified as 5-HT₂ and 5-HT₁, respectively (Berridge and Heslop, 1981) and it is interesting that methysergide and cyproheptadine are capable of blocking the serotonin-induced elevation of both cyclic AMP and calcium, albeit with different potencies. The corpus cardiacum of the cockroach has been reported to contain serotonin receptors linked to adenylate cyclase, with the profile of antagonists reported to be cyproheptadine > mianserin > cis-flupenthixol > gramine (Gole et al., 1987). A similar profile was found for cricket mandibular closer muscles (Baines and Downer, 1991), in which the rank order of potency of serotonergic antagonists was spiperone > cyproheptadine > gramine > phentolamine, suggesting the presence of receptors similar to vertebrate 5-HT₂ receptors. Studies carried out on the blood sucking bug, Rhodnius prolixus, show that serotonergic receptors on the epidermis are blocked by a number of vertebrate 5-HT₂ receptor antagonists that have a rank order of potency of mianserin > methysergide > cyproheptadine > propranolol (ineffective) (Barrett and Orchard, 1990). Thus, many insect serotonergic receptors appear to be linked to adenylate cyclase, and yet show characteristics of vertebrate 5-HT₂ receptors.

The neurally-evoked increases in cyclic AMP levels, and the effects of dopaminergic and serotonergic antagonists upon this increase, lend further support to the hypothesis that dopamine and serotonin are localized within the SN₁ and SN₂, and that receptors for these amines are present on Locusta salivary glands. It is very probable that stimulation of nerve 7b leads to a release of contents from synaptic sites or neurohaemal areas associated with SN1 and SN2 innervation of the salivary gland acinar cells. The neurally-evoked elevation of cyclic AMP in the salivary glands following extracellular stimulation suggests that the releasable contents of SN1 and SN2 bind to receptors that are linked to adenylate cyclase. The ability of the dopaminergic antagonist SCH-23390, and the serotonin antagonist spiperone, to partially inhibit the neurally-evoked elevation of cyclic AMP
confirms the previous results of neurochemistry (Gifford et al., 1991; Ali et al., 1993), that dopamine and serotonin are localised within the SN1 and SN2. However, the elevation of cyclic AMP achieved in the presence of both antagonists is an intriguing result. It is possible that this cyclic AMP accumulation is due to residual serotonin binding to receptors, since we found that no serotonin antagonist was able to completely inhibit the serotonin-induced elevation of cyclic AMP even when applied at relatively high concentrations. Alternatively, stimulation of nerve 7b may lead to a release of substances, besides serotonin and dopamine, that may also be capable of elevating cyclic AMP levels on locust salivary glands.

Our investigations into the presence of serotonin and dopamine receptors subtypes on the salivary glands of the locust have thus far led us to suggest that these amines bind to adenylate cyclase-coupled receptors that appear to be similar to vertebrate 5-HT2 and vertebrate D1 receptors. As yet we are unaware of the presence of receptors that are coupled to other second messenger systems; however, experiments are underway in order to determine this. One possibility may be the phosphatidylinositol hydrolysis second messenger system, since it appears to play a crucial role in the salivation process of the blowfly (Fain and Berridge, 1979). Results from these studies will enable us to gain a clearer understanding of the roles played by serotonin and dopamine in the salivary secretion of the locust.

References


IV. The uptake and release of serotonin and dopamine associated with locust
(Locusta migratoria) salivary glands.

Abstract
The uptake and release characteristics of dopamine and serotonin in the salivary glands of the locust, Locusta migratoria were examined. Cyclic AMP levels were determined in salivary glands in which the salivary nerve was stimulated under different experimental paradigms. Stimulation of the salivary nerve leads to time and frequency-dependent elevations of cyclic AMP levels in the glands. The potent and specific D1 receptor antagonist, SCH-23390, is capable of partially inhibiting the electrophysiologically-induced elevations of cyclic AMP. The salivary glands appear to possess uptake transporters for serotonin and dopamine. [3H] Serotonin uptake is sodium-dependent and is composed of high and low-affinity components. [3H] Dopamine uptake is sodium-independent and can be partially reduced by a high-K+ challenge and by an ice-cold saline. Uptake inhibitors are capable of blocking the uptake of radiolabelled serotonin and dopamine. There is a calcium-dependent efflux of [3H] serotonin and [3H] dopamine from previously loaded salivary glands in response to stimulation of the salivary nerve and to treatment with a high-K+ saline.
Introduction

Locust salivary glands are innervated via the salivary nerve, nerve 7b, which is a branch of nerve 7 that originates from the suboesophageal ganglion (Altman and Kien, 1979). Nerve 7b contains the axons of 2 neurons, SN1 and SN2, whose cell bodies are located within the suboesophageal ganglion (Altman and Kien, 1979). Dopamine has been shown to be present within SN1 (Gifford et al., 1991) and serotonin within SN2 (Tyrer et al., 1984; Gifford et al., 1991; Ali et al., 1993). It has therefore been postulated that dopamine and serotonin probably act as neurotransmitters in the salivary glands of the locust. However, in order to demonstrate that a particular chemical is a transmitter substance, a number of criteria must first be fulfilled. For instance, the chemical must be present within the presynaptic neuron: this has been shown for dopamine in SN1 (Gifford et al., 1991; Ali et al., 1993) and for serotonin in SN2 (Tyrer et al., 1984; Gifford et al., 1991; Ali et al., 1993). The presynaptic neuron must contain the enzymes necessary for synthesis of the proposed neurotransmitter: this is indicated by the positive tyrosine hydroxylase-like immunoreactivity of SN1 and the salivary gland processes, suggesting the presence of this rate-limiting enzyme for the production of catecholamines within SN1 (Orchard et al., 1992; Ali et al., 1993), although currently there are no data to indicate the presence of enzymes responsible for the biosynthesis of serotonin within SN2. The chemical must mimic the postsynaptic actions of the natural transmitter: there is evidence to suggest that dopamine and serotonin alter salivary secretory rates (Baines et al., 1989) and elevate cyclic AMP levels in a dose-dependent manner (Ali and Orchard, 1994), while stimulation of the salivary nerve also alters secretory rates and elevates cyclic AMP levels in the glands (Baines et al., 1989; Ali and Orchard, 1994). Drugs that modify the postsynaptic actions of the natural transmitter should have similar effects upon the actions of the chemical under investigation: we have found that specific dopaminergic and serotonergic receptor antagonists are capable of blocking the dopamine- and serotonin-induced elevation of cyclic AMP levels in the glands and that receptor blockers are capable of partially blocking the nerve 7b-stimulated increase in cyclic AMP levels in the glands (Ali and Orchard, 1994). Other criteria that must be fulfilled include evidence that
the chemical is released upon specific stimulation of the neuron and that there exists a mechanism for removal of the chemical. Therefore, if it is to be demonstrated that dopamine and serotonin are true functional neurotransmitters in this system, it must be shown that there is a removal mechanism for these amines associated with the salivary glands. Further evidence for the release of dopamine and serotonin from SN1 and SN2 is also necessary.

Vertebrates have two main mechanisms for removing and inactivating biogenic amines from the synaptic cleft; the first is a high-affinity uptake mechanism in which a transporter translocates the amine into the neuron, while the second is the use of enzymes such as monoamine oxidase (MAO) and catechol O-methyl transferase (COMT), that initiate the metabolism of biogenic amines (Cooper et al., 1991). Insects do not appear to have large quantities of MAO and it is presumed that an uptake transporter is the primary method of removal of biogenic amines from the synaptic cleft (Evans, 1980). The presence of a high-affinity uptake mechanism for serotonin is well documented for the abdominal nerves of *Rhodnius prolixus* (Flanagan and Berlind, 1984; Orchard 1989), and in cultured neurons from *Periplaneta americana* (L.) (Bermudez and Beadle, 1989). Similarly, high-affinity uptake mechanisms for octopamine appears to be present in the cockroach ventral nerve cord (Evans, 1978), and in the larval firefly light organ (Carlson and Evans, 1986).

Several of these studies have also shown that once taken up via a high-affinity mechanism, radiolabelled amines can be released from these preparations (Orchard, 1989; Carlson and Evans, 1986; Morton and Evans, 1984; Flanagan and Berlind, 1984).

The present study was carried out in order to investigate the characteristics of the release of dopamine and serotonin from SN1 and SN2, and to determine whether uptake transporters for dopamine and serotonin are present in the salivary glands of the locust.

**Materials and Methods**

*Insects*

Adult male *Locusta migratoria migratorioides* (R. & F.) were taken 6-10 days post
ecdysis from a crowded colony maintained at 30°C under a 12 h light:12 h dark regime. Insects were fed daily on freshly grown wheat supplemented with bran.

**Cyclic AMP measurements**

Salivary glands were dissected under physiological saline (150 mM NaCl; 10 mM KCl; 4 mM CaCl$_2$; 2 mM MgCl$_2$; 4 mM NaHCO$_3$; 5 mM HEPES, pH 7.2; 90 mM sucrose; 5 mM trehalose) and assayed for cyclic AMP levels after different experimental treatments. Modified salines used in these treatments included Ca$^{2+}$-free, high-Mg$^{2+}$ saline (4 mM CaCl$_2$ replaced with 8 mM MgCl$_2$), and high-K$^+$ saline (100 mM NaCl replaced with equimolar KCl). Salivary glands were incubated in 0.5 mM isobutyl methylxanthine (IBMX) for 10 min at room temperature along with the appropriate incubation saline with or without pharmacological reagents. At the end of the incubation period the reaction was terminated by the addition of 500 µl of boiling 0.05 M sodium acetate buffer, pH 6.2, followed by 5 min of boiling. The samples were stored at -20°C until cyclic AMP determinations were performed. The samples were thawed, sonicated, centrifuged at 8800g for 10 min, and the supernatant removed for cyclic AMP determination while the pellet was dissolved in 50 µl 0.5 N NaOH for protein determination. Cyclic AMP levels were determined by radioimmunoassay (Lange and Orchard, 1986) using a commercially available kit (New England Nuclear, Lachine, Quebec, Canada). The protein content of the glands was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) based upon the method of Bradford (1976) using gamma globulin as standard.

**Uptake of [³H] amine**

Salivary glands were dissected under physiological saline at room temperature and incubated in saline containing either 5-hydroxy[³H] tryptamine creatinine sulphate (6.73x10$^{11}$ Bq/mmol) or [2,5,6-³H] dopamine (7.29x10$^{11}$ Bq/mmol) (Amersham, Buckinghamshire, England). Tissues were routinely incubated in 1.85x10$^4$ Bq amine/ml for 10 min except in the time course studies and in release experiments in which longer incubation times were needed in order to assess the release of [³H] amine above background levels. For [³H]dopamine, tissues were incubated in 3.7x10$^4$ Bq amine/ml for
10 min since the effects of various treatments on the uptake of [³H]dopamine were more noticeable at concentrations above 1.85x10⁴ Bq amine/ml. In experiments designed to test the effects of ions or uptake inhibitors on the uptake of [³H]amine, tissues were washed for 30 min at room temperature in the ion free medium (except for high-K⁺ saline), or in the presence of the uptake inhibitor, prior to a 10 min incubation in [³H]amine. The various ion-free salines consisted of the following: Na⁺ replaced by Tris-HCl, for sodium-free saline; Ca²⁺ replaced by equimolar Mg²⁺, for calcium-free saline; NaCl and KCl replaced by equimolar Na acetate and K acetate, for the reduced-Cl⁻ saline; 100 mM Na⁺ replaced by 100 mM K⁺, for the high-K⁺ saline. Following incubations, tissues were washed several times for 30 min in saline in order to remove extraneous radioactivity, solubilized overnight in 0.5 ml of BTS-450 tissue solubilizer (Beckman, Mississauga, ON, Canada) and dissolved in 10 ml Econofluor (New England Nuclear). Samples were left overnight to dark-adapt and counted on a Beckman LS60001C scintillation counter. Radioactivity was estimated at a counting efficiency of 43% and correction for variations in quenching was made by reference to the external standard.

**Release of [³H] amine**

For release experiments, samples were incubated in 1.85x10⁴ Bq amine/ml, washed for 30 min and then placed in 100 µl of a series of solutions consisting of high-K⁺ saline (100 mM K⁺ replacing 100 mM Na⁺) or Ca²⁺-free, high-Mg²⁺ saline, with or without extra potassium, or placed in 200 µl of normal saline or Ca²⁺-free, high-Mg²⁺ saline, for electrical stimulation of the salivary nerve. The incubation saline from all release experiments was collected after each 5 min incubation and the radioactivity in the sample was measured. All incubation media were added directly to Ready Caps with Xtalscint (Beckman) and air dried overnight. The counting efficiency of the Ready Caps was estimated at 25% for [³H]serotonin and 16% for [³H]dopamine.

For HPLC analysis, tissues were incubated in 1.85x10⁴ Bq amine/ml for 1 h, washed for 30 min, extracted into 100 µl of ice-cold HPLC buffer (75 mM Na₂HPO₄; 0.3 mM sodium octyl sulphate; 50 µM EDTA; 8% methanol; 5% acetonitrile adjusted to pH 3.3 with orthophosphoric acid), sonicated, centrifuged at 8800g and filtered through a 0.2 µm filter
before injection. 10 µl of the extracted gland was added directly to Ready Caps and counted for radioactivity. The injected samples were spiked with 200 pg serotonin or dopamine and run on HPLC with electrochemical detection as previously described (Ali et al., 1993; Elia et al., 1994). 1 ml fractions were collected and 200 µl samples were added to Ready Caps to count the radioactivity.

**Neurophysiology**

Salivary glands were dissected and placed in 200 µl pools of saline containing the appropriate reagents. Nerve 7b was gently sucked into a suction electrode and stimulated at 15 Hz for 5 min with 1 ms square-wave pulses, except for time course and frequency experiments. A second electrode was placed distally to monitor evoked potentials (Fig 1). Voltages of 0.3-2 V were generally sufficient for recruitment of SN1 and SN2. For stimulation in a Ca²⁺-free, high-Mg²⁺ saline, glands were washed in the saline for 5 min prior to stimulation. In experiments designed to test the effects of the dopamine receptor antagonist SCH-23390 on cyclic AMP levels, salivary glands were routinely preincubated for 5 min in 10 µM SCH-23390, prior to 5 min of stimulation. Following stimulation, tissues and perfusate were added to 500 µl boiling 0.05 M sodium acetate buffer, pH 6.2, followed by 5 min of boiling. Cyclic AMP levels were determined as described above. Controls for these experiments consisted of salivary glands stimulated with a subthreshold voltage.

For release of [³H]amine, salivary glands were incubated for 1 h in [³H]amine, washed for 30 min in normal saline or 25 min in normal saline and 5 min in Ca²⁺-free, high-Mg²⁺ saline (for stimulation in a Ca²⁺-free saline). The tissues were prepared for stimulation as mentioned above and stimulated for 5 min. Each gland functioned as its own control, in which nerve 7b was stimulated with a subthreshold voltage for 5 min. The perfusate was collected on Ready Caps and fresh saline was applied to the glands. Nerve 7b was then stimulated with a supratheshold stimulus for 5 min. The perfusate was collected onto Ready Caps. Salivary glands were collected, solubilized and counted for radioactivity.
Chemicals

Imipramine, clomipramine, nomifensine, bupropion, quipazine, GBR-12909 and (+)-SCH-23390 were obtained from Research Biochemical Inc. (Natick, MA, USA). 5-Hydroxy-[G-^3H]tryptamine creatinine sulphate (6.73x10^{11} Bq/mmol) and [2,5,6-^3H]dopamine (7.29x10^{11} Bq/mmol) were obtained from Amersham, UK. Dopamine, serotonin and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistics

One way analysis of variance (ANOVA) with a Duncan's multiple range and Newman-Keuls statistical test were performed on groups of data within each experiment to ascertain which groups were significantly different from the controls (P<0.05). Values given in the text represent means ± S.E.M. and values of N are given in the figure legends.

Results

Cyclic AMP determinations

Stimulation of the salivary nerve, nerve 7b which contains one dopaminergic axon (from SN1) and one serotonergic axon (from SN2) at 15 Hz for various times resulted in an accumulation of cyclic AMP in the salivary glands. The elevated levels of cyclic AMP reached a plateau after the first 2 min of stimulation (Fig. 2A) resulting in an increase of cyclic AMP over basal levels of 53.6±10 pmol/mg protein. Salivary glands preincubated in 10 μM of the dopamine receptor antagonist SCH-23390 for 5 min experienced smaller elevations of cyclic AMP levels, reaching approximately 22 pmol/mg protein above basal levels. Such high concentrations of the dopamine antagonist have previously been shown to block completely dopamine-induced elevations of cyclic AMP levels in salivary glands, and so it is likely that the difference in cyclic AMP values between the two curves (Fig. 2A) is due to the effects of dopamine. The cyclic AMP levels in the presence of SCH-23390 are probably due to serotonin released by neural stimulation. Serotonergic receptor blockers were not used in these experiments since no blocker was completely effective at
Figure 1: A) Schematic drawing of a salivary gland from *Locusta migratoria* prepared for neural stimulation and recording from the salivary nerve, nerve 7b. B) Stimulation of nerve 7b (SA, arrow) is followed by action potentials from SN1 (second trace), SN1 and SN2 (third trace), and eventually from SN1, SN2 and the satellite nervous system (SNS) (fourth trace) as the stimulating voltage is gradually increased (from top trace to bottom trace).
Figure 2: The effect of neural activity upon the cyclic AMP content of the salivary glands. A) Time course when nerve 7b is stimulated at 15 Hz with 1 ms square wave pulses in normal saline (●) and in the presence of 10 μM SCH-23390 (■). B) Effect of frequency of stimulation when nerve 7b is stimulated for 5 min in normal saline (●) and in the presence of 10 μM SCH-23390 (■). An increase in cyclic AMP content represents the increase from control tissues. Basal levels 36.96 ± 2.38 pmol/mg protein. All values represent mean ± SEM of 6-13 determinations. An asterisk marks values that are significantly different from control values (p<0.05).
A

Increase in cAMP content (pmol/mg protein)

Time (min)

B

Frequency (Hz)
inhibiting the effects of serotonin (Ali and Orchard, 1994); thus, differences due to the serotonergic antagonist may not necessarily be an accurate reflection of the effects of serotonin.

To investigate the effects of the frequency of activity of SN1 and SN2 on salivary gland cyclic AMP levels, the salivary nerve was stimulated for 5 min at varying frequencies (Fig. 2B). Cyclic AMP levels were maximally elevated (approximately 40 pmol/mg protein) at and above a frequency of 5 Hz. Frequencies above 20 Hz were not used since it was difficult to maintain action potential activity above this frequency. In addition, the physiologically relevant level of activity of the salivary neurons rarely exceeds a frequency of 15 Hz (Baines et al., 1989), although a recent publication (Schactner and Bräunig, 1995), indicates an average frequency of activity for SN1 (during feeding) of 8-9 Hz and for the SN2 of 6-7 Hz. SCH-23390 was capable of reducing the effects of neural stimulation upon cyclic AMP levels. This was particularly noticeable at low frequencies of stimulation (Fig. 2B).

Treatment with high-K⁺ saline (50-100 mM) resulted in elevations of cyclic AMP levels within salivary glands (Fig. 3). Maximum values of 68 pmol/mg protein were achieved in 100 mM KCl. Ca²⁺-free, high-Mg²⁺ saline abolished the effect of elevated [K⁺] on cyclic AMP levels (Fig. 3). Ca²⁺-free, high-Mg²⁺ saline also blocked the increase in cyclic AMP levels induced by electrical stimulation of the salivary nerve (Fig. 4A), suggesting that this saline either inhibits the release of amines from SN1 and SN2, or blocks the cyclic AMP transduction mechanism. To test the viability of the cyclic AMP transduction mechanism, we examined the ability of dopamine and serotonin to elevate cyclic AMP levels in tissues incubated in a Ca²⁺-free, high-Mg²⁺ saline (Fig. 4B). Dopamine and serotonin (0.5 μM) were able to elevate cyclic AMP levels in the glands, suggesting that the inhibition in stimulated preparations is probably due to a requirement for Ca²⁺ to mediate the release of dopamine and serotonin from SN1 and SN2.

**Uptake of [³H] serotonin and [³H] dopamine**

Salivary glands incubated in tritiated serotonin and dopamine (0.5 μM) accumulated [³H]amine over a period of 3 h in which maximum accumulation was attained after the
Figure 3: The effect of [K'] mM (●) on the cyclic AMP content of locust salivary glands. The effect of K' in saline containing of 0 mM Ca^{2+}, 8 mM Mg^{2+} is also shown (■). Values represent mean ± SEM of 6-12 determinations. An asterisk marks values that are significantly different from control values (P<0.05).
Figure 4: The cyclic AMP content of locust salivary glands (in pmol/mg protein) is shown in response to A) neural stimulation (15 Hz for 5 min) in normal saline and in 0 mM Ca$^{2+}$, 8 mM Mg$^{2+}$ saline (bar) and in response to B) 5x10^{-7}$M serotonin (5-HT) and dopamine (DA) in 0 mM Ca$^{2+}$, 8 mM Mg$^{2+}$ saline (bar). ST, neural stimulation; CTL, control group. Values represent mean ± SEM of 7 determinations. An asterisk marks values that are significantly different from control values (P<0.05).
Figure 5: Time course of uptake of serotonin (●) and dopamine (▼) by locust salivary glands from saline containing 5x10^{-7} M amine. Values represent mean ± SEM of 5 determinations.
first 60 min of incubation (Fig. 5). For [³H]serotonin, a maximum accumulation of 2.58±0.25 pmol per salivary gland was achieved, whereas the maximum for [³H]dopamine was 1.58±0.11 pmol/sg.

To investigate the Na⁺-dependence of the uptake mechanism, glands were incubated for 10 min at room temperature in either normal or Na⁺-free (Na⁺ replaced by Tris-HCl) saline over a concentration range of 0.05 μM-50 μM [³H]serotonin (Fig. 6A) and dopamine (Fig. 6C). The Na⁺-specific uptake shown in Fig. 6A (filled triangles) is calculated by subtracting the Na⁺-sensitive uptake from the Na⁺-insensitive uptake over the complete concentration range. At lower concentrations (0.05 μM-0.5 μM) the Na⁺-specific uptake represents approximately 67% of the total uptake, whereas at higher concentrations (0.5 μM-50 μM) the Na⁺-specific uptake represents approximately 32% of total values. An Eadie-Hofstee plot of the Na⁺-specific uptake of [³H]serotonin (Fig. 6B) suggests the presence of two separate uptake mechanisms with different affinities for serotonin. The high-affinity component appears to have a maximum rate of uptake of 1.24 pmol/salivary gland/10 min and an apparent Km of 0.74 μM. The low-affinity component has a maximum rate of uptake of 7.75 pmol/salivary gland/10 min and an apparent Km of 0.3 μM.

Interestingly, the uptake of [³H]dopamine was not affected by a Na⁺-free saline, as shown in Fig. 6C. To investigate further the uptake mechanisms of dopamine and serotonin, we examined the ability of different ions to affect the uptake of [³H]amine.

Ice-cold saline, Na⁺-free, and a combined Na⁺- and Ca²⁺-free saline were capable of reducing the uptake of [³H]serotonin in locust salivary glands. The total accumulation of radiolabel in tissues incubated in 0.5 μM [³H]serotonin for 10 min was 703±29 fmol/salivary gland compared to glands incubated in ice-cold saline of 67±16 fmol/salivary gland, Na⁺-free saline of 250±29 fmol/salivary gland and in a Na⁺- and Ca²⁺-free saline of 256±46 fmol/salivary gland. Ca²⁺-free saline had no effect on the ability of glands to accumulate [³H]serotonin. Similarly, a reduced-Cl⁻ (12 mM Cl⁻ instead of 172 mM Cl⁻) and a high-K⁺ (100 mM K⁺) saline had no significant effect on the uptake of [³H]serotonin in a 10 min incubation period (Fig. 7A). The effect of ions on [³H]dopamine uptake were different from those on serotonin uptake. An ice-cold saline reduced the [³H]dopamine
Figure 6: A) The rate of uptake of serotonin and dopamine into the salivary glands (V in pmol/10 min) is plotted against serotonin ([S] in mM) and dopamine concentration in the bathing medium. The filled circles (○) represent total uptake in normal saline and the open triangles (▼) represent the Na⁺-insensitive uptake from a Na⁺-free saline. The Na⁺-sensitive uptake component, represented by the filled triangles (▼) in A, was obtained by subtracting the Na⁺-insensitive component from the total uptake rate at each serotonin concentration. Points represent mean ± SEM of 5 determinations. B) An Eadie-Hofstee plot for the Na⁺-specific uptake component of serotonin into the salivary glands. Lines were drawn using a first-order linear regression for each uptake component. High-affinity component: \( r^2 = 0.91, P < 0.05 \); low-affinity component: \( r^2 = 0.97, P < 0.05 \).
Figure 7: Effect of ions and an ice-cold saline on the accumulation of \[^3\text{H}\]amine by salivary glands from saline containing \(5 \times 10^{-7} \text{ M}\) serotonin (A), or \(10^{-6} \text{ M}\) dopamine (B). Controls (CTL) represent the accumulation of \[^3\text{H}\]amine in normal saline at room temperature for 10 min. Na\(^+\), Na\(^+\)-free; Ca\(^{2+}\), Ca\(^{2+}\)-free, Na\(^+\) Ca\(^{2+}\), Na\(^+\) and Ca\(^{2+}\)-free; Cl\(^-\), reduced-Cl\(^-\) (172 mM to 12 mM); K\(^+\), 100 mM K\(^+\); Ice, ice-cold saline. Values represent mean \pm SEM of 6 determinations. An asterisk marks values that are significantly different from control values (P<0.05).
Serotonin

\[ [\text{H}]\text{Amine uptake (fmol/10 min)} \]

A

Dopamine

\[ [\text{H}]\text{Amine uptake (fmol/10 min)} \]

B

\[ \text{CTL Na Ca Na Ca Cl K Ice} \]

\[ \text{CTL Ice Ca Na Ca Cl K} \]
Figure 8: Effect of uptake inhibitors (5x10^{-5} M) on the accumulation of [3H]amine by salivary glands from saline containing 5x10^{-7} M serotonin (A) or 10^{-6} M dopamine (B). Control (CTL) represents uptake of amine in normal saline for 10 min. Na^+, Na^-free saline; Ice, ice-cold saline; IM, imipramine; CL, clomipramine; GBR, GBR-12909; BU, bupropion; NO, nomifensine; QU, quipazine. Values represent mean ± SEM of 6 determinations. An asterisk marks values that are significantly different from control values (P<0.05).
(1 µM) uptake from control levels of 1190±119 fmol/salivary gland to 579±62 fmol/salivary gland, a reduction of 51% (Fig. 7B). The only other saline capable of affecting the uptake was high-K⁺ saline, which reduced the accumulation to 667±101 fmol/salivary gland, representing an inhibition of 44% of control levels. The effects of a number of uptake inhibitors on the uptake of [³H]serotonin is shown in Fig. 8. The specific mammalian serotonin uptake inhibitor quipazine, the tricyclic antidepressants imipramine and clomipramine, and the dopamine uptake inhibitors, GBR-12909 and nomifensine were capable of blocking the uptake of [³H]serotonin, whereas the other dopamine uptake inhibitor, buproprion, was ineffective (Fig 8A). At a concentration of 50 µM, quipazine was the most effective uptake inhibitor, inhibiting the serotonin uptake by 79% while imipramine was as effective as a Na⁺-free saline, reducing the uptake of radiolabel from control levels of 801±36 fmol/salivary gland in normal saline to 260±24 fmol/salivary gland, representing a 68% inhibition of total uptake and a 90% inhibition of Na⁺-specific uptake. Clomipramine (50 µM) was also an effective inhibitor, blocking the specific uptake of [³H]serotonin by 89%. GBR-12909 blocked 89%, and nomifensine 74%, of the Na⁺-specific uptake, whereas buproprion had no effect on the uptake of serotonin in locust salivary glands. The tricyclic antidepressants and the dopamine uptake inhibitors were all capable of reducing the uptake of [³H]dopamine (1 µM) in glands incubated in 50 µM inhibitor (Fig. 8B). The most potent inhibitor was imipramine, which inhibited the total uptake by 70%. Clomipramine had a similar potency and reduced the uptake from 1563±208 fmol/salivary gland to 534±123 fmol/salivary gland, which represents an inhibition of 66%. The selective dopamine uptake inhibitor, GBR-12909, was as effective as imipramine and clomipramine and reduced the uptake levels by approximately 67%. The other dopamine uptake inhibitors, buproprion and nomifensine, were slightly less effective, reducing the uptake levels to 965±174 fmol/salivary gland and 966±222 fmol/salivary gland respectively. The selective serotonin uptake inhibitor quipazine had no effect on the dopamine uptake rate in locust salivary glands. The effects of imipramine and GBR-12909 were not significantly different from that of ice-cold saline, in which the uptake of [³H]dopamine was reduced to 808±91 fmol/salivary gland (Fig. 8B).
Figure 9: Depolarization-induced release of serotonin (A) and dopamine (B) from salivary glands. Salivary glands were incubated in 5x10^{-7} M [^3H]serotonin or [^3H]dopamine for 1 h, washed in saline several times for 30 min and then incubated for 5 min in various solutions. There was a significant increase in the efflux of tritium induced by 100 mM K^+ saline when Ca^{2+} was present (45-50 min period) but not in the absence of Ca^{2+} (35-40 min period). A second consecutive exposure to 100 mM K^+ saline released an equivalent amount of tritium to the first exposure, in glands incubated in serotonin. No tritium was released from glands incubated in dopamine during the second exposure to 100 mM K^+. Values represent mean ± SEM of 6 determinations. Final amine contents of salivary glands was 1.86±0.13 pmol of [^3H]serotonin and 0.94±0.17 pmol of [^3H]dopamine for A and B respectively. An asterisk marks values that are significantly different from control values (P<0.05).
Depolarization-induced release of serotonin (A) and dopamine (B) from salivary glands. Salivary glands were incubated in 5x10^{-7} M [^3]H]serotonin or [^3]H]dopamine for 3 h, washed in saline several times for 30 min and then incubated for 5 min in various solutions. There was a significant increase in the efflux of tritium induced by 100 mM K^+ saline when Ca^{2+} was present (45-50 min period) but not in the absence of Ca^{2+} (35-40 min period). A second consecutive exposure to 100 mM K^+ saline released an equivalent amount of tritium from glands incubated in both serotonin and dopamine. Values represent mean ± SEM of 8 determinations. Final amine contents of salivary glands was 2.16±0.39 pmol of [^3]H]serotonin and 1.62±0.15 pmol [^3]H]dopamine in A and B respectively. An asterisk marks values that are significantly different from control values (P<0.05).
Release of \[^{1}H\] amine

To investigate the possibility that accumulated amines are cycled into a releasable pool, tissues previously incubated in tritiated amines were incubated for 5 min in various solutions consisting of 100 mM K\(^+\) or Ca\(^{2+}\)-free, high-Mg\(^{2+}\) saline with or without 100 mM K\(^+\). Salivary glands were capable of releasing previously accumulated \[^{1}H\]serotonin in response to high-K\(^+\) saline as shown in Figs 9 & 10. When salivary glands were previously loaded for 1 h in \[^{1}H\]serotonin, they were capable of releasing approximately 36 fmol of tritium above background levels when incubated in a high-K\(^+\) saline (45-50 min period compared with 40-45 min period), while a second consecutive 5 min wash in 100 mM K\(^+\) induced an efflux of 34 fmol of tritium (50-55 min period compared to 40-45 min period; Fig. 9A). The total release of tritium was 3.7% of the final content of the glands (1.86±0.13 pmol/salivary gland). Glands preincubated for 1 h in \[^{1}H\]dopamine released approximately 80 fmol of tritium above background levels in the first 100 mM K\(^+\) wash. The second consecutive 5 min 100 mM K\(^+\) wash was unable to induce an efflux of tritium above background levels: 95.3±10.2 fmol/salivary gland in the 50-55 min period compared to 98.4±21.2 fmol/salivary gland in the 40-45 min period). The total release of tritium was 8.5% of the final \[^{3}H\] content of the glands (0.94±0.17 pmol/sg).

In a similar experiment, when salivary glands were preincubated for 3 h in \[^{1}H\]serotonin, washed, and then incubated for 5 min in various solutions, approximately 120 fmol/salivary gland of radiolabel above background levels was released during the 45-50 min period (Fig. 10). A second incubation in high-K\(^+\) saline also induced about 120 fmol/salivary gland of release. Ca\(^{2+}\)-free, high-Mg\(^{2+}\) magnesium saline was capable of preventing the release of radiolabel as shown during the 35-40 min incubation period in Fig. 10A. The total release of 240 fmol/salivary gland was approximately 11.2% of the final \[^{3}H\] content of the glands (2.16±0.39 pmol/salivary gland). Similar results were obtained for glands incubated in \[^{1}H\]dopamine. A high-K\(^+\) saline was effective at inducing a release of radiolabel of approximately 66 fmol/salivary gland above basal levels (Fig. 10B). Ca\(^{2+}\)-free, high-Mg\(^{2+}\) saline abolished the effects of 100 mM K\(^+\), as shown in Fig. 10B. The release of radiolabel during the complete 10 min incubation period in 100 mM K\(^+\) was 8% of the final \[^{1}H\]dopamine accumulated by the salivary glands (1.62±0.15
Figure 11: Neurally stimulated release of serotonin and dopamine. Salivary glands were incubated in $5 \times 10^{-7}$ M $[^3]$H]serotonin (A) or $[^3]$H]dopamine (B) for 1 h, washed for 30 min in saline or 15 min in normal saline and 15 min in 0 mM Ca$^{2+}$, 8 mM Mg$^{2+}$ saline, and then nerve 7b was electrically stimulated for 5 min. Controls (CTL) consisted of glands stimulated with a subthreshold stimulus while stimulations (ST) consisted of the same glands stimulated with a suprathreshold stimulus. Efflux of tritium was also quantified in 0 mM Ca$^{2+}$, 8 mM Mg$^{2+}$ saline (bar). Values represent mean ± SEM of 5-8 determinations. Final amine contents of the salivary glands was $1.41±0.05$ pmol of $[^3]$H]serotonin and $0.97±0.13$ pmol $[^3]$H]dopamine in A and B, respectively. An asterisk marks values that are significantly different from control values (P<0.05).
Serotonin

Dopamine

[3H]Amine efflux (fmol/5 min)

A

Serotonin

no Ca\(^{2+}\)/high Mg\(^{2+}\)

B

Dopamine

no Ca\(^{2+}\)/high Mg\(^{2+}\)
pmol/salivary gland).

To examine the ability of SN1 and SN2 to release \[^3\text{H}\text{ serotonin}\] and \[^3\text{H}\text{ dopamine}\] from previously loaded stores, salivary glands were incubated in 0.5 \(\mu\text{M}\) \[^3\text{H}\] amine, washed for 30 min and then nerve 7b was electrically stimulated. The perfusate from stimulated preparations contained 39 fmol/salivary gland of \[^3\text{H}\text{ serotonin}\] and 41 fmol/salivary gland of \[^3\text{H}\text{ dopamine}\], above the levels of the non-stimulated preparations (Fig. 11A, B). These values represent 2.8% of the total \[^3\text{H}\text{ serotonin}\] content and 4.2% of the total \[^3\text{H}\text{ dopamine}\] content of the salivary glands. Similar experiments in a Ca\(^{2+}\)-free, high-Mg\(^{2+}\) saline showed no significant difference between the efflux levels of stimulated and unstimulated preparations for both amines (Fig. 11A, B).

An HPLC analysis of loaded salivary glands revealed that approximately 100±16% of \[^3\text{H}\] label accumulated from \[^3\text{H}\text{ dopamine}\] co-eluted with dopamine, while 94±9% of label accumulated from \[^3\text{H}\text{ serotonin}\] co-eluted with serotonin. These results suggest that the amines are not metabolised within the first 1.5 h after uptake is initiated (1 h incubation period plus 30 min wash).

**Discussion**

There have been a number of studies demonstrating that the biogenic amines dopamine and serotonin are associated with the salivary glands of *Locusta migratoria* (Baines *et al.*, 1989; Baines and Tyrer, 1989; Gifford *et al.*, 1991; Ali *et al.*, 1993; Ali and Orchard, 1994), where they have a number of effects, including increasing salivary secretory rates and elevating cyclic AMP levels. Receptors for serotonin and dopamine have also been shown to be associated with salivary gland preparations (Lafon-Cazal and Bockeart, 1984; Ali and Orchard, 1994). We have carried out the present study to confirm that dopamine and serotonin are indeed natural neurotransmitters in locust salivary glands. We therefore examined the release and inactivation mechanisms for these amines in the glands.

We have previously shown that stimulation of nerve 7b of isolated salivary gland
preparations leads to increases in cyclic AMP levels that can be partially inhibited by dopaminergic (10 μM SCH-23390) and serotonergic (10 μM spiperone) receptor antagonists, from which we infer that dopamine and serotonin are released from nerve terminals and subsequently interact with receptors coupled with adenylate cyclase (Ali and Orchard, 1994). In the time course experiments using stimulation at 15 Hz, it seems clear that approximately 50% of the elevated cyclic AMP level is due to the release of dopamine since this is the amount blocked by the dopamine receptor antagonist, SCH-23390. The remainder of the elevated level of cyclic AMP is probably due to the release of serotonin although this could not be definitively shown because we have not found a serotonergic antagonist which is 100% effective against serotonin (Ali and Orchard, 1994). SCH-23390, in contrast, is effective at completely blocking relatively large dopamine-induced elevations of cyclic AMP levels. The frequency experiments (Fig. 2) showed that SCH-23390 is more effective at blocking neurally-evoked increases in cyclic AMP levels when both neurons are active at lower frequencies, suggesting a greater involvement of dopamine than of serotonin, at lower frequencies. The activities of the neurons are quite different between the feeding and non-feeding states of the animal (Baines et al., 1989), and it would appear that the optimal release of dopamine and serotonin occur at different frequencies. This may not be a surprising result if, as recently postulated by Schactner and Bräunig (1995), dopamine and serotonin play different roles in the formation and secretion of saliva. Specifically, dopamine may induce the secretion of water and ions, while serotonin may induce the secretion of enzymes and proteinaceous components in the saliva (Schactner and Bräunig, 1995).

The stimulation-induced elevation of cyclic AMP levels is Ca\(^{2+}\)-dependent; however, Ca\(^{2+}\)-free saline does not interfere with the ability of dopamine and serotonin to bind to their receptors and initiate the cyclic AMP transduction mechanism, implying that Ca\(^{2+}\) is needed for dopamine and serotonin to be released from SN1 and SN2. A high-K\(^{+}\) saline which is known to depolarise neurons and induce the release of substances at synaptic and neurohaemal sites is also capable of resulting in an elevation of cyclic AMP levels in a Ca\(^{2+}\)-dependent fashion. Furthermore, both neural stimulation and a high-K\(^{+}\) medium can induce the release of [\(^{3}\)H]dopamine and [\(^{3}\)H]serotonin from previously loaded locust
salivary glands in a Ca\(^{2+}\)-dependent fashion. Taken together these results suggest that dopamine and serotonin are released from SN1 and SN2 respectively, upon electrical stimulation of these neurons.

The primary means of removing biogenic amines from the synaptic cleft is via a re-uptake mechanism which transports the amine into the presynaptic terminal. We sought to examine the possible presence of uptake mechanisms for dopamine and serotonin associated with locust salivary glands. Over a 3 h period, glands incubated in \([^{3}H]\)amine accumulated radiolabel linearly over the first hour of incubation. The re-uptake of amines has been shown on a number of occasions to be dependent upon Na\(^{+}\) (see Kanner, 1994 for review) and therefore a Na\(^{+}\)-free saline should reduce, if not abolish, the uptake of \([^{3}H]\)serotonin and \([^{3}H]\)dopamine. The uptake of \([^{3}H]\)serotonin in locust salivary glands is also Na\(^{+}\)-dependent and appears to have two different uptake rates depending on the substrate concentration. The high-affinity component occurs at lower concentrations and has an apparent Km of 0.74 µM while the lower affinity component has a Km of 30 µM. The Km of the high-affinity component (0.74 µM) is comparable to that in a lobster serotonergic neuron of 0.66 µM (Livingstone et al., 1981) and in Rhodnius of 0.22 µM (Orchard, 1989). The high-affinity uptake of \([^{3}H]\)serotonin into cultured Periplaneta neurons has a Km of 0.23 µM (Bermudez and Beadle, 1989), and of octopamine in cockroach VNC of 0.48 µM (Evans, 1978). The low-affinity uptake mechanism in Locusta migratoria salivary glands has a higher Km (30 µM) than the low-affinity mechanisms reported by Bermudez and Beadle (1989) in cockroach cultured neurons (2.14 µM) and that reported by Evans (1978) in cockroach VNC (19.8 µM). It is possible that both high and low affinity mechanisms in locust salivary glands occur at synaptic sites. However, there are also some serotonergic neurohaemal areas lying on nerve 7b (Bräunig, 1987; 1988). Thus it is possible that the neurohaemal areas are capable of accumulating \([^{3}H]\)serotonin, and releasing it in response to a high-K\(^{+}\) challenge. Since we are primarily concerned with the SN1 and SN2, our electrophysiological stimulation paradigms excluded the recruitment of these neurohaemal areas. As shown in Fig. 1, we were able to preferentially recruit activity from the SN1 and SN2 with a voltage subthreshold to that
required for the recruitment of the neurohaemal areas. This allowed us to investigate the efflux of radiolabel due to activation of the salivary neurons.

In vertebrates, uptake transporters occur on neurons as well as on glial cells and one of our concerns is the cellular location of the uptake transporter. This remains to be definitively determined, since a high-K⁺ efflux may occur from a number of cell types; however, the efflux of radiolabel from neurally-stimulated preparations strengthens our postulation that at least some of the uptake of radiolabel occurs in the salivary neurons. That the release of [³H]amine is Ca²⁺-dependent also suggests a Ca²⁺-mediated release from nerve terminals.

The uptake mechanism appears to be dependent primarily on Na⁺ since a Ca²⁺-free, reduced-Cl⁻ or a high-K⁺ saline had no significant effect on the uptake of [³H]serotonin. This ion-dependence differs from that for the uptake of [³H]serotonin in cultured neurons of Periplaneta (Bermudez and Beadle, 1989) in which a Cl⁻-free medium and a high-K⁺ saline reduced the uptake of [³H]serotonin. In locust salivary glands as in cultured neurons from Periplaneta americana, ice-cold medium reduced the uptake of radiolabel, presumably because the uptake mechanism is energy-dependent. The ability of specific uptake transporters to reduce the uptake of [³H]serotonin (Fig. 8) serves to strengthen the evidence for the presence of a serotonergic transporter. It is interesting that of the three dopamine transport inhibitors, two (GBR-12909 and nomifensine) inhibit the uptake of [³H]serotonin, whereas the other (buproprion) does not. It may be worth noting that imipramine and clomipramine are tricyclic antidepressants while nomifensine has a bicyclic structure and appears to be more closely related to the tricyclics than buproprion. Thus, it appears that the salivary gland serotonin uptake transporter has some pharmacological differences from those reported from vertebrates.

Similar experiments for [³H]dopamine gave different results. The time course of [³H]dopamine uptake by locust salivary glands showed an initial high rate followed by a levelling off of the curve. Interestingly, however, the dopamine uptake by the salivary glands is not dependent upon Na⁺ (Fig. 7). The uptake of radiolabel is also not dependent upon Cl⁻ and Ca⁺, but may depend upon ATP and a K⁺ gradient, as indicated by the reduced uptake in ice-cold and a high-K⁺ saline. Since high-K⁺ saline also depolarizes the
neurons, it is likely that the reduced uptake in 100 mM K\(^+\) may be due to a more positive membrane potential. The tricyclic antidepressants and the dopamine uptake inhibitors were all capable of reducing the uptake of \([^{3}\text{H}]\text{dopamine}\), although the specific mammalian uptake inhibitor, quipazine was ineffective. The most potent uptake inhibitors actually had a greater effect than ice-cold saline on uptake rates, suggesting that an ice-cold saline does not fully inhibit the energy-dependent uptake, or that the uptake mechanism is not solely dependent upon energy.

The release of \([^{3}\text{H}]\text{serotonin}\) and \([^{3}\text{H}]\text{dopamine}\) from the salivary glands, either by stimulation of the salivary nerve or by a high-K\(^+\) challenge, indicates that the \([^{3}\text{H}]\text{amine}\) associated with the glands is placed in a releasable pool. The trafficking of \([^{3}\text{H}]\text{amine}\) into releasable pools seems to occur via different time-dependent methods for each amine. A larger percentage of the total \([^{3}\text{H}]\text{serotonin}\) content of the glands is released from 3 h preincubated glands than from 1 h preincubated glands, although in each case equal quantities of tritium released during the first and second exposures to high-K\(^+\) saline. Tissues preincubated in \([^{3}\text{H}]\text{dopamine}\), however, release the same percentage of tritium regardless of the preincubation time. Also, the complete release of tritium occurs during the first 5 min exposure to high-K\(^+\) saline for tissues preincubated for 1 h, but it occurs in equal quantities during the first and second exposures to high-K\(^+\) saline for tissues preincubated for 3 h.

Some uptake transporters were able to inhibit the uptake of dopamine into locust salivary glands, which indicates that, in spite of being Na\(^+\)-independent, there is probably a dopaminergic transporter associated with SN1 that has some unique characteristics. In vertebrates the uptake of catecholamines appears to take place via at least two types of mechanisms with different ionic dependence, uptake_1 and uptake_2, although recently evidence has been presented for the uptake of \([^{3}\text{H}]\text{catecholamines}\) via a mechanism distinct from these two (Martel et al., 1994). These uptake mechanisms have been characterised for noradrenaline in particular, in which the uptake_1 mechanism is Na\(^+\)-dependent whereas uptake_2 is not dependent upon extracellular changes of Na\(^+\) or Ca\(^{2+}\) concentration over short periods (Bönisch et al., 1985). If, however, Na\(^+\) and Ca\(^{2+}\) are omitted from the
preincubation medium for relatively long periods (90 min), uptake becomes affected.

The present results suggest that the dopamine transporter in locust salivary glands is unique but not necessarily unusual since it may have minor similarities with the uptake system in vertebrates. Locust salivary glands may therefore provide us with an excellent opportunity to study the physical and biochemical properties of biogenic amine uptake transporters in insects.

References


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V. The effect of suboesophageal ganglion nerve stimulation, and neurotransmitters, on the activity of the salivary neurons in *Locusta migratoria*

Abstract

The salivary glands of the locust, *Locusta migratoria*, are innervated via the salivary nerve which contains the axons of two neurons: salivary neuron 1 (SN1) and salivary neuron 2 (SN2). The salivary nerve projects from the suboesophageal ganglion. Neural stimulation of the maxillary nerve with extracellular electrodes, at low frequencies (0.5-2 Hz), resulted in phase locked SN1 activity, with a latency of 13-17 ms; stimulation at higher frequencies (5-15 Hz) resulted in SN1 activity that was not phase locked with the stimulus. SN2 activity occurred rarely and was never phase locked with the stimulus. Neural stimulation of other nerves on the SOG did not lead to salivary neuron activity. Salivary neuron activity was enhanced when the SOG was isolated from the ventral nerve cord. Bath perfusion of acetylcholine and the cholinergic agonists, carbachol and nicotine, resulted in enhanced salivary neuron activity. Bath perfusion of either the muscarinic agonist, oxotremorine M, or of serotonin, dopamine, glutamate or GABA had no apparent effect on salivary neuron activity.
Introduction

Our understanding of insect behaviour depends in part upon our knowledge of motorneuron control of peripheral systems and an understanding of connectivity within the insect nervous system. Several attempts have been made toward investigating neural circuitry and connectivity in the invertebrate central nervous system (CNS) with promising results. The relative simplicity of the invertebrate nervous system coupled with the presence of well-defined identifiable neurons allows these systems to act as models in our attempts to understand the relationships between neurons and between neurons and their effector systems.

Many studies have focused on the interaction between neurons and effector systems because they are more accessible than neuron-neuron relationships. For example, the innervation of the locust ovipositor muscle and oviduct has been used to study aspects of neuromodulation and central pattern generators (Belanger and Orchard, 1993a, b, 1992; Kalogianni and Pfluger, 1992). Innervation of the flight muscles and patterns of activity in flight neurons and interneurons have also been investigated (Pearson et al., 1985).

The neurons controlling the salivary glands of the African migratory locust, Locusta migratoria provide an insect preparation that is well-suited to the study of connectivity and control of motorneuron activity concerns. The salivary glands of Locusta are innervated via the salivary nerve which contains the axons of two motorneurons whose somata are located in the suboesophageal ganglion (SOG) (Altman and Kien, 979). The somata of salivary neuron 1 (SN1) are on the dorsal surface of the ganglion and project to the contralateral salivary nerve root and thence to the salivary glands. The somata of the SN2 are located on the ventral surface of the labial neuromere and project ipsilaterally towards the salivary nerve root and on to the gland where they branche over the acini. The SN1 contain dopamine while the SN2 contain serotonin and GABA (Ali et al., 1993; Watkins and Burrows, 1989). Dopamine and serotonin are thought to be natural neurotransmitters in this system, and both are capable of inducing fluid secretion and inducing elevations of cyclic AMP levels in the glands (Ali and Orchard, 1996; Ali et al.,
Stimulation of the salivary neurons also leads to fluid secretion and elevated cyclic AMP levels in the glands (Ali and Orchard, 1994; Baines et al., 1989). The salivary neurons are primarily active during feeding, firing at 6-8 Hz for SN1 and 4-5 Hz for SN2 (Schactner and Bräunig, 1995), but in addition, the SN1 are periodically active at 5-8 Hz during interfeeding bouts while the SN2 are completely silent (Schactner and Bräunig, 1995). This suggests that the SN1 and SN2 perform separate roles, but also that there are separate control mechanisms for SN1 and SN2.

The SOG of the locust has 8 pairs of nerve roots and one single median nerve emanating from its ventral surface (Albrecht 1953). The major nerves project to the mandibles, maxillae and labial palps and contain many fine sensory axons as well as several motorneurons (Altman and Kien, 1987). Other small nerves project to the muscles of the head and neck region, including nerve 7 which branches into 7A, the salivary nerve, and nerve 7B which projects to the neck (Altman and Kien, 1979). Thus, the SOG controls the mouthparts in a manner which allows fine coordination of feeding. Since the relevant neurons are found within one ganglion, the need for interneurons is reduced and more direct contacts between neurons can occur (Altman and Kien, 1987). Locusts taste their food via sensory chemoreceptors on the maxillary and labial palps (Blaney 1981; Blaney and Duckett 1975; Blaney 1974) and begin to feed if the conditions are appropriate. The salivary neurons become active following food tasting but prior to activity in the mandibular closer muscle, muscle M9 (Schactner and Bräunig, 1995). As feeding comes to a close, the SN2 cease firing when M9 activity stops (Schactner and Bräunig, 1995), whereas SN1 is frequently active for a short period following the cessation of M9. Thus the salivary neurons probably receive several signals immediately before, and at the end of, feeding. Where do these messages come from, and which putative neuroactive substances are involved in these signals? These are the questions that are addressed in this paper.
Materials and Methods

Insects

Adult male *Locusta migratoria* were taken 3-7 days post-ecdysis from a crowded colony maintained at 30 °C under a 12 h: 12 h light: dark regime. Insects were fed daily on freshly grown wheat supplemented with bran.

Extracellular stimulation

Animals were anaesthetised with CO₂ and dissected under physiological saline (150 mM NaCl; 10 mM KCl; 4 mM CaCl₂; 2 mM MgCl₂; 4 mM NaHCO₃; 5 mM HEPES, pH 7.2; 90 mM sucrose; 5 mM trehalose) to expose the ventral surface of the SOG. The legs, wings and abdomen were removed and the thorax was cut into dorsal and ventral halves, care being taken to leave the brain and the connectives intact. The ventral half was pinned ventral side up and the mandibles, maxillae and labial palps were removed to expose the ventral surface of the SOG. The cuticle on the surface of the neck was also removed to expose the salivary nerve where it joins the salivary duct and projects to the glands. A recording suction electrode, fashioned out of glass, was used to gently draw on the salivary nerve, while a second suction electrode was used to stimulate the salivary nerve and other nerve roots on the SOG. Stimuli were delivered to the preparation from a WPI instruments Anapulse Stimulator (model 302-T). Square wave pulses (maximum voltage of 3 V and a maximum duration of 2 ms) were generally used to elicit action potentials. Stimulation of the nerve roots took place a small distance away from the body of the ganglion to ensure that only axons within that particular nerve were stimulated. Extracellular recordings from the salivary nerve were preamplified and band-pass filtered (300 Hz- 1KHz) using a WPI instruments differential preamplifier (model DAM-5A). The SN1 and SN2 were identified since the SN1 axon propagated the larger amplitude action potential (Schactner and Bräunig, 1995; Baines and Tyrer, 1989). In experiments in which only 1 axon was recorded, a brief square wave pulse of very large voltage (15 V) was passed to directly activate the salivary neurons, and the sizes of the action potentials were compared to the original recording to ascertain which neuron was active. Recordings were
Application of neurotransmitters

Prior to perfusion experiments, activity was recorded from the salivary nerve under different conditions to ascertain a control activity of the salivary neurons. Hence, the connectives were either left intact or the anterior connectives were cut, or the posterior connectives were cut, or both anterior and posterior connectives were cut while the ganglion was perfused with saline alone. For perfusion experiments, both pairs of connectives were cut and the ganglia was desheathed using fine forceps to gently pull apart the ganglionic sheath. The perfusion system consisted of a gravity feed, controlled by an intervenous wheel. The perfusion rate varied between 0.8 ml/min and 1.5 ml/min and saline was removed from the preparation dish by a suction pump.

Data was stored on a Sony Video Cassette Recorder (Model SL-HF860 D) coupled to an Analog/Digital VCR Adaptor (Model PCM-2) and fed to an RC Electronics (Goleta, CA) data acquisition system, running on an IBM compatible 386 computer. Data was acquired in the electronic chart recorder mode and analyzed using a window discriminator and histogram generating modules. To examine the effects of pharmacological agents on the salivary neurons, drugs were dissolved in physiological saline at various concentrations and applied to the preparation via the perfusion system. The preparation was perfused with saline and activity monitored from the salivary nerve for 5 min. This was followed by perfusion of the drug for 10 min, and subsequent wash off with saline for at least 10 min. In many cases the final saline wash was continued to ascertain when salivary neuron activity ceased.

Drugs

The following drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA): dopamine, serotonin, octopamine, glutamate, GABA, carbamylcholine chloride (carbachol) and acetylcholine. Oxotremorine M and nicotine ditartrate (nicotine) were obtained from Research Biochemicals Inc. (Natick, MA, USA).
Statistics

Student's t-tests were performed on groups of data for preparations which were perfused with neurotransmitters to ascertain which groups were significantly different from the control preparations (isolated SOG perfused with saline) (P<0.05).

Results

Extracellular stimulation of either contralateral or ipsilateral maxillary nerves frequently resulted in SN1 activity in the salivary nerve (8 out of 12 preparations)(Fig 1). On several occasions the SN1 spike was phase-locked with maxillary nerve stimulation and occurred between 13 and 17 ms following the stimulus artefact (4 out of 8 preparations)(Fig 1). In rare instances an SN2 spike was recorded, but was never phase locked with the stimulus artefact or with SN1 activity. Thus, SN1 is silent prior to stimulation, becomes active during stimulation, and ceases firing when stimulation ceases. At low frequencies (0.5-2 Hz), SN1 is usually phase-locked with the stimulus and exhibits a 1:1 correspondence; however, as the frequency of stimulation is increased, the 1:1 correspondence is lost and activity occurs randomly during the stimulation regime. As a general rule, stimulation of other nerve roots did not elicit activity in the salivary nerve, however, on rare occasions (2 out of 12 preparations) stimulation of the mandibular nerves led to salivary nerve activity. Care was taken in interpreting results since cutting any connective pair led to an increase in activity of the salivary neurons (see later). Frequently, spontaneous activity of the salivary neurons began following cutting the connectives. Varying the stimulation paradigm for the maxillary nerve had no apparent effect on the occurrence of SN1 activity. For instance, stimulation with single square wave pulses or with trains of pulses led to very similar results. Stimulation of both maxillary nerves, simultaneously, also resulted in SN1 activity (n=7) which was phase locked at low frequencies (0.5-2 Hz) and was random at higher frequencies (5-15 Hz). Stimulation of either anterior or posterior connective(s) usually did not lead to SN1 activity except on some occasions (3 out of 9 preparations).
Figure 1: Schematic drawing of a suboesophageal ganglion showing the relative positions of SN1 and SN2. Stimulating (S) and recording (R) suction electrodes are shown. B) Extracellular trace from the salivary nerve in response to stimulating the ipsilateral maxillary nerve. C) Expanded trace of B of a stimulus (arrow) and SN1 spike recorded from the salivary nerve. Scale bars: B) 60 μs, 0.13 mV; C) 10 ms, 0.2 mV.
Figure 2. Effects of bath application of saline on an isolated SOG. (A) Extracellular recording from the salivary nerve. Saline was perfused throughout the entire experiment. 1 min, 10 min and 20 min represents the times at which the 3-sec, sample traces were taken. (B), (C) Frequency histogram of SN1 and SN2 firing. Ganglia were isolated from the CNS at time t=0 min (arrow). Each bin represents the number of action potentials per 25 s.
Recording from Salivary Nerve: Controls

To further investigate the activity of the salivary neurons under various conditions, an attempt was made to record from the salivary nerve with the connectives left intact to the brain and thoracic ganglia, or with the connectives cut to partially or completely isolate the SOG. The salivary neurons were silent when the SOG was left connected to the CNS in 8 of 11 preparations. In only 3 of 11 preparations were the salivary neurons active (SN1: 0.9 ± 0.7 Hz; SN2: 0.5 ± 0.4 Hz). However, if either pair of anterior or posterior connectives (data not shown), or both, were cut then the salivary neurons became active after disconnection (Fig 2). The onset of activity of the SN1 and SN2 varied in time and the SN1 were usually active at a slightly higher frequency than SN2. Once active, SN1 and SN2 fired continuously, sporadically in some cases, for the duration of the experiment (up to 40-50 min).

Application of Neurotransmitters

Since the salivary neurons appeared to be inhibited if the SOG was left connected to the CNS, we decided to fully isolate the SOG and test the effects of neurotransmitters. Ganglia were desheathed and putative neurotransmitters applied by perfusion to the preparation. The resulting activity (if any) was compared to the background activity elicited by the salivary neurons of an isolated SOG in a saline-perfused preparation. Bath application of acetylcholine (ACh; 10^{-3} M) did not appear to increase the activity in SN1 over that obtained from an isolated SOG (Fig 3); however, SN2 activity was significantly enhanced over SN2 activity in saline-perfused isolated SOG (P<0.05). The average activity of the SN1 during bath application to an isolated SOG was 1±0.1 Hz, and of SN2 was 1.1±0.1 Hz (n=8). Such high concentrations of ACh were used since acetylcholinesterase activity in insects appears to be extremely high, plus the concentration of ACh attained at synaptic sites from bath application is likely to be very much less than the bath concentration. In addition, neurotransmitter concentration at synaptic sites is known to reach levels in the mM range (Clements 1996). To further investigate the possibility of the presence of cholinergic receptors associated with the salivary neurons, a non-hydrolisable ACh analog, carbamylcholine chloride (carbachol), was tested for its
Figure 3. Effects of bath application of $10^{-3}$ M acetylcholine on an isolated SOG. (A) Application of acetylcholine appeared to enhance the firing rate of SN1 and SN2, although only SN2 activity was significantly different from controls ($P<0.05$). The effects of acetylcholine were reversed after several mins of washing in saline. (B), (C) Frequency histogram of SN1 and SN2 firing. Arrow denotes the time at which the ganglia were isolated from the CNS. Each bin represents the number of action potentials in 25 s.
A

Saline

Acetylcholine

SNS

Saline wash

B

$10^{-3}$ M ACh

SN1

Events/25 s

0 5 10 15 20 25

Time (min)

Cut

C

$10^{-3}$ M ACh

SN2

Events/25 s

0 5 10 15 20 25

Time (min)

Cut

0.2 mV

0.5 s
Figure 4. Effects of bath application of $10^{-4}$ M carbachol on an isolated preparation. (A) Application of carbachol significantly enhanced the firing rate of SN1 and SN2 ($P<0.05$). The effects of carbachol on SN2, but not SN1, were reversed after several mins of washing in saline. SN2 activity was slightly higher than average in this preparation. (B), (C) Frequency histogram of SN1 and SN2 firing. Arrow denotes the time at which the ganglia were isolated from the CNS. Each bin represents the number of action potentials in 25 s.
A Saline

Carbachol

Saline wash

B

10^{-4} M Carb

Events/25 s

Time (min)

C

10^{-4} M Carb

Events/25 s

Time (min)
ability to activate the salivary neurons. Carbachol was capable of increasing the activity of both SN1 and SN2 over control preparations (Fig 4) (P<0.05). The average frequency of SN1 was 6.5 ± 0.9 Hz, and of SN2 was 2.6±1.2 Hz. The onset of SN1 activity usually occurred within 4-6 min and SN2, 4-7 min. The effects of carbachol were either difficult to wash off, or induced an activity level in SN1 which was sustained by other means. SN2 Activity decreased to a low level during wash off of carbachol, or in some preparations ceased altogether. Carbachol was used at a lower concentration than ACh since it is resistant to breakdown by acetylcholinesterase. Since carbachol was capable of enhancing the activity of the salivary neurons, other ACh analogs, such as oxotremorine M and nicotine were tested. Oxotremorine M is a specific muscarinic agonist, whereas nicotine specifically activates nicotinic receptors. Oxotremorine M did not appear to enhance the activity of SN1 or SN2. The average frequency of SN1 was 1.8±0.4 Hz (n=10), and SN2, 0.4±0.2 Hz (n=10): values which are not significantly different from the control preparations. Nicotine had very different effects and enhanced the activity of SN1 and SN2 (P<0.05) with an average firing rate of 8±1.3 Hz in SN1 (n=6) and 5.8±0.8 Hz in SN2. Most notable were the transient effects of nicotine since spiking of the salivary neurons only occurred for 2-3 min at high frequencies (Fig 5). Also of note was the increased activity of the SN2 which occured during the final wash in 4 out of 6 preparations.

SN1 and SN2 possess overlapping dendrites and it was speculated that dopamine, serotonin or GABA might have an effect on one or other salivary neurons since dopamine is found in the SN1 and serotonin and GABA in the SN2. Hence, these neuroactive substances were tested for their ability to affect the firing of SN1 or SN2. Bath application of dopamine, serotonin or GABA failed to enhance the activity of SN1 or SN2 over the control preparations which were disconnected from the CNS. There was usually activity in the SN1 and SN2, however the activity began at different times for each preparation, remained at low levels throughout the experiment (0.2-1 Hz), and in the majority of cases prolonged washing did not change the activity. The average activities for SN1 and SN2 during bath application of dopamine, serotonin and GABA were as follows: Dopamine, SN1: 0.9±0.5 Hz; SN2: 0.4±0.5 Hz (n=9); serotonin, SN1: 1.4±1.1
Figure 5. Effects of bath application of $10^{-4}$ M nicotine on an isolated SOG. (A) Application of nicotine transiently increase the firing of SN1 and SN2 ($P<0.05$). During washing in saline, SN2 is periodically active at high frequencies while SN1 is not. (B), (C) Frequency histogram of SN1 and SN2 firing. Arrow denotes the time at which the ganglia were isolated from the CNS. Each bin represents the number of action potentials in 25 s.
A
Saline

Nicotine

Saline wash

B

10^{-4} M Nic

SN1

Events/25 s

Time (min)

C

10^{-4} M Nic

SN2

Events/25 s

Time (min)

0.2 mV

0.5 s
Figure 6. Effects of bath application of $10^{-4}$ M octopamine on an isolated SOG. (A) Application of octopamine does not significantly enhance SN1 activity over control preparations. SN2 activity is significantly enhanced ($P<0.05$) over controls. (B), (C) Frequency histogram of SN1 and SN2 firing. Arrow denotes the time at which the ganglia were isolated from the CNS. Each bin represents the number of action potentials in 25 s.
Saline

Octopamine

SN1

Saline wash

SN2

B

$10^{-4}$ M OA

SN1

Events/25 s

Time (min)

Cut

C

$10^{-4}$ M OA

SN2

Events/25 s

Time (min)

Cut

0.2 mV

0.5 s
Hz; SN2: 0.8±0.6 Hz (n=11); GABA, SN1: 0.6±0.3 Hz; SN2: 0.5±0.1 Hz (n=10).

The effects of glutamate were also tested since this chemical has been shown to be a neurotransmitter in insects (Benson 1993). Glutamate was incapable of inducing activity in the salivary neurons that was different from the control preparations. The average SN1 firing rate was 0.4±0.3 Hz (n=9). SN1 was silent in several preparations. SN2 commenced firing anywhere from 2-14 min following the start of bath application of glutamate and usually continued firing for 15-25 min after bath application. The average firing rate of SN2 was 0.7±0.2 Hz (n=9). This pattern of activity was not significantly different from the control preparations.

Octopamine was tested since it has been shown to be a possible neurotransmitter in several systems in the locust (Orchard et al., 1993; Orchard 1982), but also since it is present in the salivary glands and is contained in an unpaired neuron in the metathoracic ganglion that projects to the glands through transverse nerve collaterals (Bräunig et al., 1994). The activity in transverse nerves appears to be related to the activity in the salivary neurons (Baines and Tyrer, 1989), implying a possible neurotransmitter role for octopamine. In the presence of octopamine (10^−4 M) the average activity level in SN1 of 0.6±0.3 Hz (n=8) was not significantly different from control, isolated preparations (Fig 6), whereas SN2 activity (1.1±0.2 Hz; n=8) was significantly different from controls (P<0.05). The onset of SN1 or SN2 activity was not consistent and varied from preparation to preparation.

Discussion

It has been proposed that the role of the SOG in Orthoptera is twofold: firstly it serves to coordinate "higher" order activities with the brain, such as walking and flight; and secondly, it has local control over mouthparts and feeding mechanisms (Altman and Kien 1987). Feeding is a complex process whereby food must be recognised, tasted, and accepted (or rejected) in concert with the activation of mouthpart muscles and the inhibition of other forms of behaviour. The present study was undertaken in order to
investigate the control of the salivary neurons and their relationship to other neurons which innervate the mouthparts.

Several types of sensilla have been identified on virtually all the mouthparts of the desert locust *Schistocerca gregaria* (Thomas 1966). The maxillary palps of locusts are inundated with sensory receptors, many of which are chemoreceptors. Indeed, it appears that there are approximately 370 contact chemoreceptor hairs on the tip of each maxillary palp which are capable of responding to a wide range of substances (Blaney 1974; Blaney and Chapman 1969). The function of the palps appears to be primarily that of taste and food palatability; they largely determine the acceptance or rejection of food (Blaney and Chapman 1970). Bernays and Chapman (1974, in Blaney and Duckett 1975) suggest that continuous palpation may be one of the inputs necessary for feeding, but additional chemosensory input from other mouthpart sensilla may also be needed (Blaney and Duckett, 1975). The mandibles are responsible for crushing the food and are driven by activity in the mandibular closer muscle M9 (Seath, 1977). The muscles of other mouthparts follow, and move in synchrony with, the mandibles (Seath 1977). Of particular interest in this study is the activity of the salivary neurons whose onset of firing occurs about 30 sec prior to M9 activity (Schactner and Bräunig 1993).

The salivary neurons seem to be under inhibitory influences from elements outside the SOG. Since the neurons become more active when either anterior or posterior connectives are cut it is possible that ascending and descending inhibitory pathways impinge upon them, although this has yet to be verified. The significance of ascending inhibition is unclear but the neuronal pathways and connections seem to be present. We do know that the median-transverse system is linked to the salivary neurons since activation of the median nerves of the pro- and mesothoracic ganglia activate SN1 but suppress SN2 (Baines and Tyrer 1989). In addition, an octopaminergic unpaired neuron in the metathoracic ganglion innervates the salivary glands through collaterals that are connected to the median-transverse system. Thus if octopamine plays a role in salivation, it is probable that there is coordination of activity between this neuron, the peptidergic neurons that project to the glands from the pro- and mesothoracic ganglia, and the salivary neurons.
Stimulation of the maxillary palps leads to firing of the SN1 which follows with a constant latency at low frequencies. Stimulation of other nerve roots does not lead to similar results. A 1:1 correspondence between stimulus and action potentials at low, but not at high frequencies, and a 15-20 msec latency suggests a polysynaptic pathway between maxillary nerve and salivary neurons. Since the animal tastes its food through palpation prior to the onset of feeding, it may need to lubricate its mouthparts once the contact chemoreceptors in the maxillary palps have responded favourably to food, hence, the activation of the salivary neurons.

An increase in activity of the salivary neurons in response to bath perfusion of acetylcholine, carbachol or nicotine suggests the presence of cholinergic receptors somewhere in the pathway for activation of these neurons. Carbachol is a non-hydrolisable analog of acetylcholine and since it is not acted upon by acetylcholinesterases it can be perfused at a lower concentration than ACh. Furthermore, the effects of carbachol in this preparation appear to last for a longer period of time than the effects of ACh. To gain further insight into the nature of the cholinergic receptors, a muscarinic agonist, oxotremorine M, and a nicotinic agonist, nicotine, were applied to the preparation. Oxotremorine M appeared to have little effect on the salivary neurons while nicotine was very effective. The strong response to nicotine is transient which is probably due to desensitization of the receptors. Desensitization of nicotinic receptors is a well documented phenomena in several other preparations (Sattelle 1980). These preliminary results suggest the presence of cholinergic receptors, more specifically, nicotinic-like receptors. However, the absence of muscarinic-like receptors is not a certainty. Indeed, if the nicotinic receptors are desensitized within 2-3 min of activation, whereas carbachol is effective for a much longer period, then there may well be other cholinergic receptors present. Certainly, additional agonist and antagonist receptor studies must be performed in order to more fully characterize the cholinergic receptors. The exact location of these receptors in still unknown, but ACh is generally considered to be the neurotransmitter found in afferent neurons (Sattelle 1980). It is possible, therefore, that the receptors may be associated with the salivary neurons, or with neurons that impinge upon them. This remains to be determined.
The actions of acetylcholine and its agonists on insect neurons have been investigated in detail. Carbachol, bath perfused onto the desheathed ganglia of the cockroach, is capable of depolarizing neurons within the ganglion, but only when perfused at $10^{-4}$ M or higher (Sattelle et al., 1976). Furthermore, in this preparation, ACh has a threshold of ganglionic depolarization above $10^{-3}$ M, which is due to the high activity of acetylcholinesterases, since the threshold for ACh action is reduced a thousandfold in the presence of the ACh-ase inhibitor, eserine (Sattelle et al., 1976). In larval Manduca the principal planta retractor motoneuron (PPR) which is responsible for the reflexive withdrawal of the abdominal prolegs is responsive to carbachol, nicotine and oxotremorine M (Trimmer and Weeks 1989). High concentrations of carbachol and nicotine caused a depolarization and an increase in spiking of PPR, and an eventual blockade of all activity (Trimmer and Weeks 1989). Interestingly, bath application of ACh, even at $10^{-2}$ M, was ineffective; however, in the presence of the AChase inhibitor, neostigmine, ACh was capable of eliciting a depolarization of PPR (Trimmer and Weeks 1989). Even though the muscarinic agonist, oxotremorine M was ineffective on Locusta salivary neurons, it caused an increase in the firing rate of neurons in the ventral and dorsal nerve cord of Manduca when bath applied at low concentrations (Trimmer and Weeks 1989). Bath application of muscarinic agonists in the cockroach CNS were without effect (Blagburn and Sattelle 1987), although a separate study on a ventral giant interneuron in the sixth abdominal ganglion of Periplaneta americana suggests the presence of muscarinic receptors (Le Corronc and Hue 1993). Pulse application of ACh, nicotine, muscarine and oxotremorine M to the soma of locust thoracic neurons in culture reveal the presence of separate nicotinic and muscarinic receptors (Benson 1992).

Several of the putative neuroactive substances tested in this study were not capable of affecting the frequency of salivary neuron activity in isolated ganglia. However, since extracellular recordings were made from the salivary nerve, it is possible that the salivary neurons may have experienced changes in membrane potential which did not lead to the production of action potentials. An examination of isolated salivary neurons would give a clearer account of the presence or absence of aminergic or peptidergic receptors associated
with these neurons. The effects of these transmitters on insect neurons have been reported in other studies. For instance, serotonin is known to have several different effects on thoracic ganglia neurons in *Locusta*, including initiating inward currents due to a decrease in K⁺ conductance and an increase in Na⁺ conductance (Bermudez et al., 1992). In the blood feeding bug, *Rhodnius prolixus*, serotonin is capable of increasing the frequency of activity of dorsal unpaired median neurons (DUM) in the metathoracic ganglionic mass (MTGM) when bath applied at 10⁻⁶ M (Cook 1995). Dopamine depolarizes the prothoracic common inhibitory motorneuron in the cockroach, *Periplaneta americana* (Davis and Pitman 1991) with a threshold of activity of 10⁻⁴ M when bath applied. This is unlike the DUM neurons in *Rhodnius prolixus* where bath application of 10⁻⁶ M dopamine was capable of affecting their firing rate (Cook 1995). Another putative neuroactive substance capable of affecting locust motorneurons is octopamine. Bath application of mM concentrations of octopamine induced long-lasting depolarizations and bursting in a locust flight interneuron (Ramirez and Pearson 1991). Similarly, bath application of mM concentrations of octopamine increased the frequency of spontaneous spiking in octopaminergic DUM neurons and also depolarized the flexor motorneurons in the locust *Schistocerca gregaria* (Parker 1996). However, octopamine had decidedly different effects on the DUM neurons in *Rhodnius prolixus* and greatly decreased the firing rate of these neurons (Cook 1995). Even though glutamate is considered to be primarily a neuromuscular transmitter in insects, it has a more central role and receptor sites have been located on neurons in the CNS (Benson 1993). Glutamate is able to hyperpolarize the somata of isolated neurons in *Periplaneta* (Walker et al., 1981) and in *Schistocerca* (Usherwood et al., 1980). The responses of insect neurons to GABA appear to be classical in the sense of being mediated by a Cl⁻ current (Lees et al., 1987; Benson 1988a,b) through activation of a GABAₐ receptor. Thus, GABA tends to hyperpolarize the cell membrane, but its effect on the locust salivary neurons were not conclusive.

*Locusta* salivary neurons appear to be under the influence of inhibitory inputs from both ascending and descending pathways, and also can be influenced by neurons whose axons are present within the maxillary nerve. Somewhere within these connections, there are
cholinergic-like receptors which when activated induce an increased firing of SN1 and SN2. Further studies are needed to investigate the connections involved in activation of the salivary neurons, but we have begun making headway into understanding the neural circuitry underlying as complex a behaviour as feeding.

References


VI. Immunohistochemical localization of tyrosine hydroxylase in the ventral nerve cord of the stick insect, *Carausius morosus*, including neurons innervating the salivary glands.

Abstract

The distribution of tyrosine hydroxylase-like immunoreactive neurons is mapped in the ventral nerve cord of the stick insect, *Carausius morosus*. This study also examines the tyrosine hydroxylase- and serotonin-like immunoreactive elements in the salivary glands of *Carausius morosus*. Tyrosine hydroxylase is the first and rate-limiting enzyme in the pathway for the production of catecholamines, therefore tyrosine hydroxylase-like immunoreactive neurons are likely to contain catecholamines. Approximately 225 tyrosine hydroxylase-like immunoreactive neurons are present in the ventral nerve cord. The majority of these neurons appear to be interneurons. The suboesophageal ganglion contains the only unpaired neuron and the only pair of peripherally projecting tyrosine hydroxylase-like immunoreactive neurons in the ventral nerve cord. The peripherally projecting neurons project to the salivary glands via the salivary nerve. Each neuron in this pair is termed the salivary neuron 1. The remaining tyrosine hydroxylase-like immunoreactive neurons in the ventral nerve cord are interneurons and exhibit a characteristic distribution within the thoracic and the abdominal ganglia. Serotonin-like immunoreactivity is also present in the salivary glands. Positive staining of the suboesophageal ganglion for serotonin-like immunoreactivity indicates the presence of several neuron pairs including a large pair along the ventral posterior midline that project to the salivary glands via the salivary nerve. Each neuron in this pair is termed the salivary neuron 2. Backfilling of the salivary nerve with cobalt chloride reveals the presence of only two neurons within the suboesophageal ganglion that project to the salivary glands; these neurons are the salivary neurons 1 and 2. Reverse phase high performance liquid chromatography coupled with electrochemical detection of ventral
nerve cord and salivary gland homogenates confirms the presence of dopamine and serotonin.
Introduction

Biogenic amines are present throughout the nervous system of a variety of insects (Evans 1980; Brown and Nestler 1985; Nässel 1988), although their precise physiological roles are poorly understood. A necessary first step in the investigation of the role of biogenic amines in the insect nervous system is a study of their neuronal localization. To this end, immunohistochemical methods have been used with relative success as a means of locating biogenic amines in insect nervous tissue (Vieillemaringe et al. 1984; Davis 1985; Flanagan 1986; Orchard 1990). Antibodies have not only been limited to the amine of interest, but have also been made against enzymes in the pathway for the production of amines: for example, the enzyme tyrosine hydroxylase (TH) (Orchard et al. 1992; Elia et al. 1994;). Tyrosine hydroxylase is the first and rate limiting enzyme in the biochemical pathway for the production of catecholamines, and positive tyrosine hydroxylase-like (TH-like) immunoreactivity is indicative of the presence of catecholamines. Insects tend to produce little or no adrenaline (Evans 1980), and noradrenaline levels in insect nervous tissue are also relatively low (Evans 1980). It is therefore likely, that positive T.H.-like immunoreactivity indicates the presence of dopamine.

Dopamine is a major biogenic amine and neurotransmitter candidate in the ventral nerve cord and brain of insects (Evans 1980). When injected into the bee brain, dopamine affects the animal’s response to conditioned stimuli (Mercer and Menzel 1982). In addition, the iontophoretic application of dopamine into the mushroom body of the bee brain reduces evoked potentials after antennal stimulation (Mercer and Erber 1983). More recently, studies on a prothoracic common inhibitory motorneuron in the cockroach has shown that dopamine can depolarize and excite the cell body of this neuron (Pitman and Flemming 1985; Pitman and Davis 1988; Pitman and Baker 1989; Davis and Pitman 1991). Dopamine also appears to play a role as a neurotransmitter in the salivary glands of the cockroach (House and Ginsborg 1985) and locust (Ali et al. 1993; Baines and Tyrer 1989). In Periplaneta americana and Locusta migratoria, the only peripherally projecting putative dopaminergic neurons from the ventral nerve cord (VNC) are the salivary
neurons, SN1 (Orchard et al. 1992; Elia et al. 1994). The cell bodies of the SN1 in these two species are located in the suboesophageal ganglion (SOG) and they project to the salivary glands via the salivary nerve (Altman and Kien 1979; Gifford et al. 1991). Dopamine alters the salivary secretion rate and the electrical response of the cockroach acinar cells (see House and Ginsborg 1985 for review). In locusts, dopamine elevates cyclic AMP levels in the salivary glands (Ali et al. 1993) and also increases the rate of salivary secretion (Baines and Tyrer 1989).

In the present study we have mapped presumptive dopaminergic neurons within the VNC of the stick insect, Carausius morosus, as a necessary prelude to further studies involving the physiological role of dopamine in this insect. In addition we have specifically investigated the nature of the innervation of the salivary glands via the salivary nerve, since insect salivary glands have proven to be useful preparations for the study of aminergic innervation. We have also investigated the innervation of the salivary glands of C. morosus as part of a larger study in which neuronal patterns of innervation will be used as characters in a phylogenetic analysis of some members of the lower Neopteran orders of insects, since the phylogenetic tree for these orders is still largely unresolved.

**Materials and Methods**

*Animals*

Adult female Carausius morosus were used from a parthenogenic colony maintained at room temperature, high relative humidity under a 12 h light: 12 h dark regime, and fed a variety of green foliage including varieties of ivy and Ficus.

*Immunohistochemistry*

Immunohistochemistry was performed on isolated VNCs and salivary glands (n = 24) which were dissected under physiological saline (15 mM NaCl; 18 mM KCl; 50 mM MgCl₂; 7.5 mM CaCl₂; 184 mM Glucose; Tris-HCl 2 mM, pH 6.6), and fixed in 2%
paraformaldehyde in Millonig's buffer for 1 h. The ganglia were desheathed, and then the
tissues were washed several times in phosphate buffered saline (10 mM phosphate buffer,
pH 7.2 containing 0.9% NaCl) for 4-6 h, then incubated in 4% Triton X-100 in
phosphate-buffered saline for 1 h at room temperature. Preparations were then processed
for either T.H.-like or serotonin-like immunoreactivity. For T.H.-like immunoreactivity,
tissues were incubated for 48 h at 8°C in a mouse monoclonal antibody generated against
tyrosine hydroxylase (Incstar Corp., Stillwater, MN, U.S.A.) diluted 1:400 in phosphate-
buffered saline containing 0.4% Triton X-100, 2% normal goat serum and 2% bovine
serum albumin. The tissues were washed for 6-8 h in phosphate-buffered saline, then
incubated for 24 h at 8°C in a 1:200 dilution of fluorescein isothiocyanate (FITC)-labelled
goat anti-mouse immunoglobulin G (Jackson Immunoresearch Labs, West Grove, PA) in
phosphate-buffered saline containing 10% normal goat serum. Preparations were then
washed in phosphate-buffered saline for 24 h, mounted and viewed in 5% n-propyl gallate
in glycerol, pH 7.3.

Serotonin-like immunoreactivity of the SOG and salivary glands (n = 14) was
investigated using similar techniques. The primary antiserum was a 1:1000 dilution of a
rabbit anti-serotonin antiserum (Incstar Corp.) and the secondary antibody was a goat anti-
rabbit immunoglobulin G labelled with FITC.

Double labelling using both the T.H. antibody and the serotonin antiserum was also
performed (n = 4). The two primaries were applied together while the secondaries were
applied sequentially. Goat anti-mouse immunoglobulin G labelled with FITC was used to
detect T.H.-like immunoreactivity while goat anti-rabbit immunoglobulin G labelled with
Texas Red was used to detect serotonin-like immunoreactivity.

Controls were performed in which the serotonin primary antiserum was preincubated
with serotonin conjugated to BSA for 18 h at 4°C. Tyrosine hydroxylase was not
available to preincubate with the T.H. primary antibody.

Mounted preparations were viewed on a Zeiss STANDARD microscope equipped with a
IV F1 epi-fluorescence condenser, camera lucida for STANDARD microscopes and an
MC 63 photomicrographic camera for 35 mm film (Carl Zeiss, D-7082 Oberkochen,
Germany).
Backfilling

Suboesophageal ganglia were dissected \((n = 21)\), removed from adult insects and placed in a pool of physiological saline in a small dish. The cut end of the salivary nerve was draped over a well of mineral oil and placed in a pool of distilled water for approximately 1 min after which it was exposed to a solution of 6% \(\text{CoCl}_2\). The preparation was covered and incubated for 36-48 h at 10°C. Following incubation, the ganglion was washed in saline and the cobalt was precipitated with a solution of ammonium sulphide (1 drop of concentrated ammonium sulphide in 8 ml of saline). The ganglion was washed several times in fresh saline and fixed in acetic acid:ethanol (1:4) for 10 min. Silver intensification was achieved according to the procedure of Davis (1982). The ganglion was mounted and preparations were drawn by using a camera lucida.

Electrochemical detection of dopamine and serotonin

Quantification of dopamine and serotonin was performed on isolated ganglia and salivary glands \((n = 5)\) using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (Orchard 1990; Elia et al. 1994). Tissues were dissected under physiological saline, isolated and placed in 100 \(\mu\)l of HPLC buffer (75 mM \(\text{NaH}_2\text{PO}_4\), 0.3 mM sodium octyl sulfate, 50 \(\mu\)M EDTA, 5% methanol and 3.5% acetonitrile adjusted to pH 3.3 with orthophosphoric acid). Tissues were sonicated, centrifuged at 8800g and filtered through a 0.2 \(\mu\)m filter before injection onto the HPLC column. Samples were injected onto a Brownlee ODS-Spheri 5 HPLC column and eluted compounds were detected electrochemically. Dopamine and serotonin levels were quantified using the external standard method.

Results

Immunohistochemistry

Suboesophageal ganglion

The suboesophageal ganglion (SOG) contains about 23 cells (11 paired and 1 unpaired)
which stain positively for T.H.-like immunoreactivity (Fig. 1). The most intensely stained cells are a pair of lateral neurons that lie anteriorly and dorsally and that project to the salivary glands (Fig. 2a). These cells are approximately 80-85 μm in diameter and are the largest as well as the most intensely stained cells in the VNC of *C. morosus*. In addition, these cells are the only cells of the VNC that project to the periphery. The salivary neurons, (SN1), possess a gross morphology that is almost identical to the salivary neurons in the cockroach and locust that also stain positively for T.H. (Ali et al., 1993; Elia et al., 1994). The axon of each of the SN1 projects to a ventral region of the ganglion where it travels contralaterally, then projects posteriorly a short distance before reversing direction to travel toward the salivary nerve root (Fig. 2a, b). A branching pattern of the SN1 can be ascertained in some preparations. A branch projects from the main axon as it begins to traverse the SOG contralaterally. The process then projects along the lateral margin of the SOG that is contralateral to the exit of the main axon into the salivary nerve. Anteriorly to each SN1 is a cluster of 3 brightly stained neurons that appear to project toward the midline of the SOG (Fig. 1). The axons branch repeatedly but cannot be traced for any great distance. Lying just ventrally to the SN1 are three cells of a smaller diameter (20 μm), two of these may be paired while the third is the only unpaired neuron in the VNC of *C. morosus* that stains for T.H. Posterior to the SN1 are 3 bilateral pairs of cells that stain with a weak intensity (Fig. 2c). Their axons could not be traced in any preparation. Along the posterior lateral margins of the SOG are 2 bilateral pairs of cells that also stain with a weak intensity.

A posterior median neuron (PMN) pair is present on the ventral surface of the ganglion (Fig. 1, 2d). Axons from these cells travel anteriorly a short distance then outwards towards the lateral margin of the ganglion. The axons project to the dorsal surface where they branch into two or three branches, of which the main branch then projects through the posterior connectives.

*Thoracic ganglia*

All immunoreactive cells in the prothoracic ganglion are on the ventral surface. The most intensely stained pair of cells is the posterior median neurons (PMN) (50 μm in
Figure 1: Distribution of T.H.-like immunoreactivity in the suboesophageal ganglion (SOG). The salivary neurons (SN1) are on the dorsal surface of the SOG while the remaining cells are on the ventral surface. SLNv, salivary nerve; PMN, posterior median neuron; SN1, salivary neuron 1. *Bar:* 400 μm.
Figure 2: a Tyrosine hydroxylase-like immunoreactivity in the SOG showing the salivary neuron cell body (white arrow) and its axon (black arrow). Bar: 100 μm. b Suboesophageal ganglion, salivary duct (open arrow), salivary nerve with SN1 axon (long arrow) and cell body of the SN1 (short arrow). Bar: 100 μm. c Suboesophageal ganglion showing various cell bodies including the posterior median neurons (arrow). Bar: 100 μm. d Posterior median neurons in the SOG. Bar: 100 μm. e Anterior SOG showing the SN1 and branching pattern of T.H.-like immunoreactive fibres (arrow). Bar: 100 μm.
diameter). The axon from each PMN leaves the cell body from the posterior surface, projects dorsally and circles the cell body to project to the anterior of the cell where it branches into three processes (Fig. 3, 4b). One process exits the ganglion via the ipsilateral posterior connective while another projects anteriorly and contralaterally. The last process projects inward to the mid ganglion where it cannot be traced any further.

In the anterior margin of the ganglion there lies a cluster of 5-6 cells (anterior cluster, ACL, Fig. 4a)(20 μm in diameter), one of which is usually set apart from the others and lies closer to the root of the anterior connectives. Cells of this cluster project their axons dorsally in one common tract that runs posteriorly and circles outward and finally into the anterior portion of the posterior lateral neuropile where it branches repeatedly. A second cluster of bilaterally symmetrical neurons lies towards the midganglion and consists of about 6 cells that project laterally toward the ventral association centre (VAC) where they appear to form dendritic branches (Fig. 3).

Immunoreactive staining in both the meso- and metathoracic ganglia is essentially the same as staining in the prothoracic ganglion. Briefly, there is an anterior cluster of about 5 cells that is situated just laterally to the anterior connective, and, as in the prothoracic ganglion, there is usually 1 cell that lies slightly apart from the cluster towards the anterior connectives (Fig. 3). These cells appear to project posteriorly to circle around, and then into the posterior lateral neuropile. There is a second cluster of 5 or 6 cells that appear to project towards the VAC (Fig. 4c, d). The mesothoracic ganglion contains a posterior lateral neuron pair (PLN) (Fig. 3, 4c). On rare occasions, a similar pair of neurons can be seen in the posterior region of the metathoracic ganglion which is fused with the first abdominal ganglion, however the staining is very faint. Due to the capricious nature of the staining of these neurons, they are depicted as a open circles in Fig. 3.

**Abdominal ganglia**

In the stick insect the first abdominal ganglion is fused with the metathoracic ganglion so that the first visibly separate ganglion is the second abdominal ganglion (Abd 2). There are five other abdominal ganglia (Abd 3-7), plus a terminal fused ganglion (Term Abd/Abd 8). Only one pair of cells repeatedly stains within Abd 2. The pair is bilaterally
Figure 3: Distribution of T.H.-like immunoreactivity in the thoracic ganglia. All cells are on the ventral surface of each ganglion. Open circles in Metathoracic ganglion represents capricious nature of these cells. PMN, posterior median neurons. Bar: 400 μm.
Figure 4:  
a Tyrosine hydroxylase-like immunoreactivity in the prothoracic ganglion showing the anterior cluster (ACI). Bar: 100 μm. 
b Prothoracic ganglion showing posterior median neuron (thick arrow) and its accompanying axons (thin arrow). Bar: 100 μm. 
c Mesothoracic ganglion showing posterior lateral neuron (arrow). Bar: 100 μm. 
d Metathoracic ganglion showing the median cluster (arrow) projecting to neuropile region. Bar: 100 μm. 
e Fifth abdominal ganglion showing 3 lateral neurons (LN) projecting to neuropile region. Sixth and seventh abdominal ganglia exhibit similar T.H.-like immunoreactive staining patterns. Bar: 60 μm. 
f Fourth abdominal ganglion showing only 1 lateral neuron and neuropile region (arrow). Bar: 60 μm. 
g Terminal abdominal ganglion showing lateral neurons (arrow). Bar: 100 μm.
symmetrical and is situated posterior to the nerve roots (Fig. 5). Abd 3 and Abd 4 usually contain 2 bilaterally paired cells, however, on occasion only one pair of cells is detected within these ganglia (Fig. 4f, 5). Abd 5-7 contain 3 bilaterally paired cells: the most posterior pair is always the more brightly stained. Projections from the posterior pair travel anteriorly a short distance before turning internally toward the centre of the ganglion. They appear to project towards the lateral VAC where the axons cannot be traced any further. The other two pairs of cells also appear to project internally to the central neuropile (Fig. 4e, 5).

Tyrosine hydroxylase-like immunoreactivity of the terminal ganglion is confined to 5 pairs of bilaterally symmetrical cells that lie along the lateral margin (Fig. 4g, 5). The most anterior and posterior pairs of cells stain brightly while the other pairs stain less intensely. There are no peripherally projecting cells. However, there are approximately 10-11 axons that enter the ganglion via the connectives. Once inside the ganglion the axonal projections are very weakly stained and on rare occasions the majority of these axons can be traced to the centre of the ganglion where they fade out of view.

**Salivary glands**

The salivary glands of *C. morosus* are composed of groups of cells in bundles or acini. The acini are attached to ducts through which saliva is secreted into larger ducts that merge together to eventually form the primary salivary duct which runs along the ventral surface of the thoracic cavity. The duct projects along either side of the suboesophageal ganglion toward the mouth. The salivary nerve leaves the ganglion, joins the duct almost immediately and runs along the duct to the glands. Tyrosine hydroxylase-like immunoreactivity of the SN1 axon is always present within the salivary nerve and can usually be seen branching over the gland forming immunoreactive processes on the acini (Fig. 6c-e). Even though T.H.-like immunoreactivity is always present in the SN1 cell body and axon, it is occasionally absent over the acini.

Distinct neurohaemal processes of immunoreactivity are present along the length of the salivary duct (Fig. 6f, g). The immunoreactive fibres are very small, lined with blebs of immunoreactive material and can be traced anteriorly of the suboesophageal ganglion and
Figure 5: Distribution of TH-like immunoreactivity in the abdominal ganglia. Abd, abdominal ganglion; Term Abd, terminal abdominal ganglion. Bar: 100 μm.
along the duct within the salivary gland.

**Serotonin immunoreactivity of the SOG and salivary glands**

The salivary glands of the stick insect stain positively for serotonin-like immunoreactivity. Fine processes of serotonin-like immunoreactivity can be seen forming a meshwork over the acini (Fig. 6a). This immunoreactivity appears to originate from a serotonergic axon within the salivary nerve (Fig. 6b) that can be traced back to the SOG. Serotonin immunoreactivity of the SOG is always weak but several pairs of cells can usually be seen including a large pair along the midline of the posterior ganglion on the ventral surface. The serotonergic axon cannot be traced once it enters the ganglion, however these cells are the same size and location as the SN2 that are filled with cobalt during the backfilling procedure (see later). Double labelling of the salivary glands reveals the presence of 2 distinct axons within the salivary nerve (data not shown). In addition, double labelling of the SOG clearly indicates that of the two salivary neuron pairs, only the SN1 stain for T.H.-like immunoreactivity and only the putative SN2 stain for serotonin-like immunoreactivity with no apparent colocalization within either pair of neurons (data not shown).

**Cobalt filling of the salivary nerve**

Cobalt filling of the cut end of the salivary nerve towards the suboesophageal ganglion reveals the presence of two cell bodies within this ganglion that project into the salivary nerve. The first somata is situated anterior, dorsal and contralateral to the filled nerve (Fig. 7). This cell has been labelled the SN1, as mentioned previously, since its morphology is very similar to that of the SN1 in the locust and the cockroach. Cobalt filling reveals a number of fine processes not revealed by T.H.-like immunoreactivity. These include a dendritic branch off of the main axon as it begins to traverse the ganglion from the contralateral side, as well as a number of fine dendrites sprouting from the main axon. The second cell revealed by backfilling is also a very large cell, approximately 85-90 μm in diameter. It is situated close to the midline of the ganglion on the ventral surface and projects ipsilaterally to the salivary nerve root and on toward the salivary
Figure 6: a Serotonin-like immunoreactivity in the salivary gland acini of *C. morosus*. Arrow indicates 1 axon of the network of serotonergic fibres ramifying throughout the acini. *Bar*: 100 μm. b Serotonin-like immunoreactivity of the SN2 axon within the salivary nerve (*arrow*) running adjacent to the salivary duct (SD) and just anterior to the salivary gland acini. *Bar*: 100μm. c Tyrosine hydroxylase-like immunoreactive axons within the salivary nerve (*arrow*) as it branches and runs adjacent to the salivary duct. *Bar*: 100 μm. d Tyrosine hydroxylase-like immunoreactivity of the salivary glands showing the main axon (*white arrow*) along the duct and smaller fibres within the acini (*black arrow*). *Bar*: 100 μm. e Salivary gland acini showing network of T.H.-like immunoreactive fibres (*black arrows*). *Bar*: 100 μm. f, g Tyrosine hydroxylase-like immunoreactive fibres (*arrows*) on the salivary duct of *C. morosus*. *Bar*: 60 μm.
Figure 7: Camera lucida drawing of cobalt backfilled salivary nerve showing the SN1 and SN2 in the suboesophageal ganglion. The SN1 cell body is on the dorsal surface of the ganglion and its unique branching pattern is shown. The SN2 cell body is on the ventral surface of the ganglion. SLNv, salivary nerve; SN1, salivary neuron 1; SN2, salivary neuron 2. Bar: 200 µm.
gland via the salivary nerve (Fig. 7). This cell does not appear to have a large or intricate branching pattern, but does send a few processes off the main axon; we have called this neuron, salivary neuron 2 or SN2.

**HPLC coupled with electrochemical detection**

The dopamine, and for comparative purposes, serotonin content of the various tissues were quantified using HPLC coupled with electrochemical detection (Table 1). Samples were spiked with dopamine and serotonin to confirm the presence of these amines associated with the various tissues. The salivary glands possess the greatest amount of dopamine (25.54 ± 1.07 pmol per gland pair) amongst the tissues analyzed. The amount of serotonin in the salivary glands was approximately 40% the dopamine content at 10.12 ± 1.34 pmol per gland pair.

**Discussion**

Tyrosine hydroxylase is the first and rate limiting enzyme in the pathway that converts the amino acid tyrosine into catecholamines (Evans 1980). Positive T.H.-like immunoreactivity is therefore a good indicator of the presence of catecholamines. This is corroborated by studies that have compared T.H.-like staining to catecholamine staining ascertained by methods such as glyoxylic acid fluorescence (Orchard 1990; Elia et al. 1994). Tyrosine hydroxylase-like immunoreactivity in insects is indicative of the presence of dopamine, since adrenaline is rarely found in insects and noradrenaline levels are relatively low (Evans 1980). Thus, T.H.-like immunoreactivity has been used with relative success to identify putative dopaminergic neurons.

Tyrosine hydroxylase-like immunoreactivity of the ventral nerve cord of the stick insect *C. morosus*, shows many features in common with T.H.-like staining in locust and cockroach (Orchard et al. 1992; Elia et al. 1994). One of these features is that the only peripherally projecting neurons from the VNC that stain for T.H.-like immunoreactivity are a pair of neurons whose somata are located within the suboesophageal ganglion and
Table 1: HPLC-EC analysis of VNC and salivary glands of the stick insect, *C. morosus*, for dopamine and serotonin content. Values represent the mean ± SEM of 5 determinations.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pmol</th>
<th>Pmol</th>
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<tr>
<td></td>
<td>dopamine/tissue</td>
<td>serotonin/tissue</td>
</tr>
<tr>
<td>Suboesophageal ganglion</td>
<td>1.86 ± 0.28</td>
<td>1.86 ± 0.19</td>
</tr>
<tr>
<td>Prothoracic ganglion</td>
<td>1.20 ± 0.13</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>Mesothoracic ganglion</td>
<td>1.11 ± 0.15</td>
<td>0.92 ± 0.14</td>
</tr>
<tr>
<td>Metathoracic ganglion</td>
<td>0.93 ± 0.28</td>
<td>1.11 ± 0.35</td>
</tr>
<tr>
<td>Abdominal ganglia</td>
<td>2.31 ± 0.29</td>
<td>2.28 ± 0.27</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>25.54 ± 1.07</td>
<td>10.12 ± 1.34</td>
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which innervate the salivary glands. This pair of neurons exhibits gross morphological features similar to a pair of salivary neurons in both *L. migratoria* and *P. americana*, and we have called this pair, the SN1. HPLC-EC analysis of the salivary glands and suboesophageal ganglion confirms the presence of dopamine associated with these tissues, making a strong case for the dopaminergic content of the SN1. The nature of the T.H.-like immunoreactive staining on the acini suggests that dopamine probably acts as a neurotransmitter at these sites. We were interested as to the extent of innervation of the salivary glands from the SOG and thus sought to identify the presence of other salivary neurons within this ganglion. Cobalt filling of the salivary nerve reveals the presence of only two pairs of neurons in the SOG; the first are the SN1, while the second are an equally large cell pair (90 μm in diameter) termed SN2. The SN2 in locusts are serotonergic and therefore we investigated the possible serotonergic content of the SN2 and salivary glands in *C. morosus*. The salivary glands stain intensely for serotonin-like immunoreactivity and a single stained axon is also evident within each salivary nerve. Double labelling of the salivary glands and SOG for T.H.-like and serotonin-like immunoreactivity clearly shows the presence of two separate axons within each salivary nerve, thereby eliminating the possibility of colocalization of dopamine and serotonin within the SN1. The weak serotonin-like immunoreactivity of the SN2 cell bodies is in stark contrast to the strong immunoreactivity of their axons and nerve endings. A possible explanation for this may be that serotonin is made and stored in greater quantities closer to its site of release at the glands than in the cell body.

The innervation of the salivary glands of *C. morosus* is very similar to that of *L. migratoria* and *P. americana*. The salivary glands in these animals receive dopaminergic innervation from the SN1 (Gifford et al. 1991; Ali et al. 1993), while the SN2 of *Carausius* and *L. migratoria* are serotonergic. On the other hand, the SN2 of *P. americana* are not serotonergic but the salivary nerves contain several small diameter axons that do stain for serotonin-like immunoreactivity (Davis 1985). The salivary glands of *P. americana* also receive serotonergic innervation from the stomatogastric nervous system (Davis 1985). Locust salivary glands have receptors for both amines coupled to a
cyclic AMP second messenger system (Ali and Orchard 1994), and serotonin and
dopamine both induce increases in the salivation rate (Baines et al. 1989). Serotonin and
dopamine also alter the salivary secretion rate and the electrical response of cockroach
acinar cells (House and Ginsborg 1985). It is therefore likely, given the similar patterns
of salivary gland innervation and the relatedness of these insects, that dopamine and
serotonin would have similar effects on the salivary gland of C. morosus.

Interestingly, the salivary ducts of C. morosus appear to be innervated, whereas those of
L. migratoria and P. americana are not. The network of T.H.-like immunoreactive
neurohaemal processes along the length of the duct suggests that dopamine may be
released from these sites and may therefore influence the activity of the salivary duct cells.
This may not be particularly surprising since salivary duct cells need to regulate water
content and water loss in these animals. Indeed, even though the salivary ducts of L.
migratoria do not appear to be directly innervated, the salivary duct cells are capable of
experiencing an increase in cyclic AMP levels in response to applications of either
serotonin or dopamine (unpublished observations).

Apart from the SN1, T.H.-like immunoreactivity in the VNC of C. morosus is limited to
that of interneurons, since no other peripheral projections were seen. The staining of the
suboesophageal ganglion is similar to the T.H.-like immunoreactivity of the SOG in P.
americana and L. migratoria (Orchard et al. 1992; Elia et al. 1994). All three animals
have the SN1 plus an anterior cluster of 3 cells that lie close to each salivary neuron.
They also have lateral neurons and a posterior median neuron (PMN) pair. In C. morosus,
L. migratoria and interestingly enough Rhodnius prolixus (Orchard et al. 1992; Orchard
1990), the PMN display a similar, unique branching pattern, in which the neurons project
anteriorly a short distance, branch in the SOG, then project posteriorly to traverse the
entire length of the nerve cord.

The staining pattern among the thoracic ganglia of C. morosus is very similar, with an
anterior (ACL) and median cluster (MCL) in all three ganglia. The prothoracic ganglion
also contains a posterior median neuron pair that appears to be homologous with the
posterior median neurons in L. migratoria, P. americana and R. prolixus (Orchard 1990;
There is one unpaired neuron present in the VNC of *C. morosus* that stains for T.H.-like immunoreactivity. This neuron lies on the ventral surface of the SOG and appears to be homologous to the unpaired median neuron in the SOG of *P. americana* as shown by previous workers (Baker and Pitman 1989; Elia *et al.* 1994). There are also ventral unpaired median (VUM) neurons in *R. prolisxus* and *Drosophila melanogaster*, although *L. migratoria* appears to lack such positively stained neurons (Budnick and White 1988; Orchard 1990; Orchard *et al.* 1992).

A major thrust of our work is the role played by biogenic amines in insect behaviour and physiology. This study lays the foundation for future research on the role of dopamine within *C. morosus*. In particular the sizes, locations and accessibility of the SN1 and SN2, coupled with the fact that they are the only two neurons that innervate the salivary glands from the suboesophageal ganglion, make this an excellent comparative system to study the effects of dopaminergic and serotonergic innervation.

References


VII. Immunochemical staining of tyrosine hydroxylase (TH) -like material in the salivary glands and ventral nerve cord of the cockroach, *Periplaneta americana* (L.)

Abstract

This study examines the immunochemical staining of TH-like material in neurons of wholemount preparations of suboesophageal, thoracic and abdominal ganglia and salivary glands from immature and adult male cockroaches. A pair of neurons (SN1) in the suboesophageal ganglion, previously shown to contain catecholamines, stain intensely for tyrosine hydroxylase-like material and are the only neurons identified which have a peripheral target (the salivary glands). The distribution of neurons containing TH-like immunoreactive material does not precisely overlap the distribution previously found for catecholamines using histofluorescence techniques. The axons of neurons which contain tyrosine hydroxylase-like material in thoracic and abdominal ganglia appear to confine themselves to the central nervous system, thus suggesting that they probably function primarily to communicate and coordinate activity within the central nervous system. All neurons identified as containing TH-like material in the ventral nerve cord and the suboesophageal ganglion are bilaterally symmetrical. Of treatments used to enhance immunochemical staining of tyrosine hydroxylase-like material, only prior injection of animals with pargyline appeared to marginally improve resolution of positive neurons.
Introduction

The biogenic amines dopamine, noradrenaline, and serotonin have been shown to be present in insect nervous tissue (Evans, 1980; Brown and Nestler, 1985; Nassel, 1988) although their physiological roles are poorly understood. Methods of identifying aminergic neurons have relied heavily on radioenzymatic assays and the glyoxylic acid histofluorescence technique. However, immunohistochemical methods that detect the presence of particular amines or of specific enzymes in the synthetic pathway of these amines are now being used more frequently and reliably (Klemm et al., 1985; Konrad and March, 1987; Budnik and White, 1988; Barrett and Orchard, 1990; Orchard, 1990; Watson, 1992). One enzyme specifically targeted is tyrosine hydroxylase (TH), the first and rate limiting enzyme in the pathway for the production of catecholamines in vertebrates (Cooper et al., 1991). Owen and Bouquillon (1992) have recently provided evidence which supports a synthetic pathway for dopamine in insects involving TH, and the gene for TH has been cloned in Drosophila (Neckameyer and Quinn, 1989). Also, other evidence indicates a correlation between the location of TH, sites previously shown to stain positively with glyoxylic acid histofluorescence, and the presence of dopamine in the nervous system (Budnik and White, 1988; Flanagan, 1984; Orchard, 1990; Nyhof-Young and Orchard, 1990; Gifford et al., 1991; Owen and Bouquillon, 1992; Orchard et al., 1992).

The cell correspondence between neurons which were immunopositive for TH-like material and catecholamine containing neurons leaves little doubt as to its specificity. However, any method available to supplement or enhance immunostaining for TH-like material would improve visual resolution of TH-containing neurons and strengthen the confidence in results from this technique. Enhancement, like silver-intensification of cobalt filled neurons, could also serve to help identify putative areas of dendritic overlap with other identified neurons.

To date, investigations which have used pargyline or forskolin to enhance TH-immunostaining have only been carried out in vertebrates (Haykal-Coates et al., 1991;
Leviel et al., 1991). Both reports point out that the increase in staining occurs without an increase in enzyme quantity. Instead, it was suggested that pargyline somehow increases the number of "available epitopes" on the TH enzyme in tissue that is aldehyde-fixed (Coates et al., 1991). The addition of forskolin, on the other hand, may result in the phosphorylation of inactive TH protein which then renders it more recognizable by the TH antibody (Leviel et al., 1991). Other studies have revealed the possibility of enhancing enzyme activity and content in noradrenergic neurons by applying reserpine, a substance known to deplete stores of dopamine and noradrenaline (Reis et al., 1974; Sorimachi, 1975; Sloley and Owen, 1982).

We are interested in connectivity within the nervous system and interaction of aminergic and peptidergic neurons. Thus, identifying the putative transmitters that neurons use to communicate with other cells, and the morphology of these neurons, are some of the goals of our research. Comparison of results generated in an independent laboratory using the glyoxylic acid method (Baker and Pitman, 1989; Gifford et al., 1991) with our immunocytochemical methods brings us one step closer to resolving which neurons contain catecholamines and thus probably dopamine.

In the study described herein we have used the technique of immunohistochemistry to identify putative tyrosine hydroxylase-containing neurons in wholemounts of the ventral nerve cord and salivary glands of immature and mature cockroaches (Periplaneta americana (L.)), while addressing the question of enhancement of TH staining in preparations treated with various agents.

Materials and Methods

Insects

Immature animals (1-1.5 cm length) and adult male cockroaches (Periplaneta americana (L.)) were taken from a colony maintained at about 25°C with light:dark cycle (12:12), and provided with food (rat chow and rolled oats) and water ad libitum. Immature
animals were not used in experiments involving enhancement of staining for TH-like material.

**Immunohistochemistry**

Ventral nerve cords and the paired salivary glands were dissected under physiological saline (Elia and Gardner, 1990) and fixed in 2% paraformaldehyde in Millonig’s buffer (Tsang and Orchard, 1991) for 1-1.5h, washed in phosphate buffered saline (PBS - 10mM phosphate buffer, 0.9% NaCl, pH 7.2) then processed for immunohistochemistry. Injection of fixative followed by dissection (under fixative) did not result in better preservation of tissues or staining of TH-like material. Tissues were incubated in a 1:300 dilution of a mouse monoclonal antibody for TH (Incstar Corp., Stillwater, MN) made up in PBS containing 2% bovine serum albumin, 2% normal goat serum and 0.4% Triton X-100. After 48-72 hours at 4°C the tissues were washed in PBS prior to incubation for 18h at 4°C in a 1:200 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS containing 10% normal goat serum. Tissues were then washed overnight in PBS, mounted between coverslips in 5% n-propyl gallate in 90% glycerol (pH7.3) and viewed using a Zeiss fluorescence microscope.

The usual control of pre-incubating the primary antiserum with the complementary antigen could not be performed because of the lack of commercially available tyrosine hydroxylase. However, specificity of staining was checked by processing tissue in the described fashion except that either the primary or secondary antiserum was omitted from the procedure. Under these conditions no staining of nervous tissue or salivary glands occurred.

*Treatments to Enhance Immunochemical Staining of TH-like Material*

The doses of drugs and incubation times used were determined with reference to previously published works (pargyline, Haykal-Coates, 1991; reserpine, Sloley and Owen (1982), Omar et al. (1982), Baker and Pitman (1989); forskolin, Leivel et al. (1991)). Our intention was to reduce, but not eliminate, the amount of dopamine in the nervous
system, or, as previously reported, increase the number of epitopes available for antibody binding (and thus increase staining (Haykal-Coates et al. (1991)). Decreasing the dopamine content of neurons should increase the activity or concentration of tyrosine hydroxylase, as the cell attempts to recover from the deficit.

Pargyline (25, 50, or 100μg in 5μl saline, (n=4, 2, 2, respectively) - Sigma Chemical Co., St. Louis, MO) was injected intra-abdominally into mature animals 1.5h or 2h prior to the beginning of fixation. In an additional test for enhancement of staining, gamma-butyrolactone (GBL) (0.75μg in 2μl - Sigma Chemical Co.) was injected 1.5h after 25 μg pargyline but 30min before the beginning of fixation (n=3).

Reserpine (10μg in 1μl glacial acetic acid - Sigma Chemical Co.; n=4) and forskolin (0.02μg in 1μl DMSO - Sigma Chemical Co.; n=4) were injected intra-abdominally about 24h prior to the beginning of fixation. Appropriate glacial acetic acid (n=4), DMSO (n=4), and saline controls were run in parallel (simultaneously) with each treated group. An equal number of saline controls were run with each treated group for assessment of enhancement by two investigators using a "double blind" technique. In addition to these preparations, 10 other saline controls were run independently.

**HPLC**

Quantification of dopamine and serotonin in salivary glands was performed using reverse-phase high performance liquid chromatography (HPLC) coupled with an electrochemical detector (Orchard, 1990; Lange et al., 1988). Although noradrenalin has been reported to be present in low concentrations in insect nervous system (Dymond and Evans, 1979; Pitman, 1985), it was not determined here for technical reasons. Briefly, salivary glands were dissected under physiological saline and placed in 100μl of HPLC buffer consisting of 75 mM NaH₂PO₄, 0.3 mM sodium octyl sulphate, 50μM EDTA, 8% methanol and 5% acetonitrile adjusted to pH 3.3 with orthophosphoric acid. The mixture was sonicated, centrifuged at 8800 xg and filtered through a 0.2μm filter before injection. Samples were injected onto a Brownlee ODS-Spheri 5 HPLC column and eluted compounds were detected electrochemically. Dopamine and serotonin levels were quantified using the external standard method. The protein content of the salivary glands
was determined using the Bio-Rad protein assay based upon Bradford (1976) using gamma globulin as standard.

**Results**

**General**

The ventral nerve cord of *Periplaneta americana* is composed of a series of linearly arranged ganglia connected by paired interganglionic connectives. In the head and thorax are the supra- and sub-oesophageal ganglia and three thoracic ganglia, respectively. The abdominal region of the ventral nerve cord contains six distinct ganglia. During embryogenesis the first abdominal ganglion fuses with the third thoracic ganglion, so that the first discrete abdominal ganglion in the adult is actually the second abdominal ganglion of the embryo (Shankland, 1975). Similarly, the sixth or terminal abdominal ganglion of the adult is thought to be a composite of embryonic neuromeres from abdominal segments 7 to 11. Nerves that innervate the salivary glands emerge from the suboesophageal ganglion (SOG) and from the stomatogastric (stomodaeal) nervous system. These are termed the salivary duct nerve and the oesophageal salivary nerve, respectively (Whitehead, 1971).

The distribution and position of somata and axons in the ventral nerve cord and SOG staining positive for TH-like material was compared in immature and adult male animals. Interestingly, the positions of stained cells and the range of intensities of staining of cells observed within a particular ganglion were the same in both immature and adult animals. For example, the salivary neurons (SN1) and the posterior-median neurons (PMN) were always the most intensely stained cells in the SOG (Fig. 1 and 2). Similarly in the SOG, one pair of the 4 pairs of cells that normally accompany the SN1 somata always stained more intensely than the remainder, in both immature and adults.

Also consistent between immature and adult animals was the bilateral symmetrical pairing of all somata in the nervous system (not including the brain) which stained positive for TH-like material, and the pattern of staining over the salivary glands. Thus,
Figure 1: Photograph of the suboesophageal ganglion (SOG) from an immature animal stained for tyrosine hydroxylase (TH). Most intensely staining for TH-like material are the salivary neurons (straight arrows (SN 1, Fig. 2)), the posterior median neurons (arrowheads - slightly out of focus (PMN)), and one of the cells in the posterior lateral cluster (curved arrow (PLCl)). Scale bar: 200μm.
Figure 2: Composite camera lucida drawings of cockroach SOG (antero-ventral view). The filled somata in the anterior (SN1) and mid-posterior (PMN) regions of the ganglion stained most intensely for TH-like material. Somata and axons with dotted outlines (e.g., two cells in the posterior lateral cluster (PLCl)) were the most lightly stained, although they were always present. A single axon was observed to travel from each SN1 to the contralateral salivary duct nerve (SDN), which was then traced to the salivary glands. No other peripherally directed axons were seen. (ACl, anterior cluster of cells which were located adjacent to SN1s; AX, axon from SN1 to SDN; C, interganglionic connectives; CO, circumoesophageal connectives (to supraoesophageal ganglion); LNP, lateral neuron pair). Scale bar: 500μm.
results noted below apply to both immature and adult animals, except for those concerning enhancement of staining by drug pretreatment (which refer to only mature animals).

SOG and Salivary Gland

The two SN1 neurons, which lie in the ventral/anterior region of the ganglion and which have previously been identified as containing the catecholamine dopamine (Gifford et al., 1991), were the largest (40-50 μm diameter in adult animals) and most intensely stained of the cells immunopositive for TH-like material (straight arrows, Fig. 1). Axons from the somata of these neurons run a short distance posteriorly before abruptly turning and crossing the ganglion to the contralateral side (Fig. 2). In most preparations the axons could be traced to their exit from the ganglion via the salivary duct nerves and along the SOG-prothoracic connectives adjacent to the main salivary duct (Fig. 3).

A cluster of 4 small (10-20 μm diameter) bilaterally paired somata (ACl - Fig. 2) anterior to the SN1 neurons always stained positive for TH-like material, although the position of these cells as a group was variable. For example, occasionally one of the clusters was observed just posterior to the accompanying SN1 neuron. Individual cells within a group were not identified. In some preparations, an axon from one of these cells was observed to cross to the contralateral side of the ganglion and then proceed in an anterior direction into the circumoesthesophageal connective. Axons from other cells in this group could not be traced.

A posterior-ventral median pair of cells (PMN, 25-30 μm diameter) close to the midline of the SOG stained with an intensity similar to that observed for the SN1 (Fig. 1 and 2). It was difficult to follow the axons of this pair of cells although they could occasionally be seen to branch within the SOG and project posteriorly into the SOG-prothoracic connective. There are several other paired somata in the SOG which were immunopositive for TH-like material although the intensity of staining of these cells was usually lighter than that of the SN1 and PMN (Fig. 2). These include the lateral neuron pair (LNP), the posterior lateral cluster (PLCI) of 3 bilaterally paired cells, and a very lightly staining pair of cells situated just posterior to the SN1 neurons (dotted outlines in Fig. 2). Upon reaching the salivary glands, the TH-positive axon from each SN1 branches
Figure 3: Photomontage of the suboesophageal ganglion and salivary duct nerve from a single preparation showing the path taken (thin arrows) by the axon from a salivary neuron (SN1). Note that the SN1 axons cross-over (thick arrow) just posterior to their cell bodies and exit into the contralateral salivary duct nerve. The outline of the salivary duct nerve is not distinct because of its close proximity to the interganglionic connective and salivary duct. Where the salivary duct splits (posterior to SD), the salivary axon bends away from the midline to follow the reservoir duct to the salivary gland. Scale bar: 200μm.
into many smaller fibres which ramify throughout the glands (Fig. 4a,b) and eventually
give rise to a fine network of processes over the acini (Fig. 4c). TH-like material was
also seen in very fine branches of the axons which extend to and lie over the surface of
the salivary gland reservoirs (Fig. 4d).

**Thoracic Ganglia**

The first thoracic ganglion (T1) contained 5 pairs of cells (all ventral) which were
immunopositive for TH-like material. Most prominent are a pair of anterior median cells
(AMN, Fig. 5a) whose axons could be followed as they cross over to the contralateral
hemisphere of the ganglion (Fig. 6a), and a posterior-median pair of cells (PMN, Fig. 5a
and 6a). The axons of the anterior-median cells appear to project into the posterior
thoracic interganglionic connectives. Other cells were evident in the median and posterior
portion of T1 (MC1 and LN) which did not stain as intensely for TH-like material
although their presence and characteristic position was always recorded (Fig. 5a and 6a).

The second thoracic ganglion (T2) contained a single bilateral pair of ventral neurons
(PLN) which were immunopositive for TH-like material (Fig. 5b and 6b). Axon tracts of
cells originating in ganglia other than the mesothoracic ganglion were usually observed
coursing through this ganglion. Although branches from these axons within T2 were not
observed, the possibility remains that they might be present but did not contain enough
TH-like immunoreactive material to be identified.

In the posterior region of the third thoracic ganglion (T3), a cluster of three, bilaterally
paired cells (PLCl) stained positive for TH-like material (Fig. 5c and 6c). Two pairs are
situated ventrally, while the most posterior pair is situated more dorsally. The axons of
these cells could not be traced with certainty although it appears that they may send axons
into the posterior interganglionic connectives, as well as supplying local interneurons
within T3.

At least four axons staining positive for TH-like material could be seen in each hemi-
connective joining the ganglia of the ventral nerve cord, both thoracic and abdominal.
The smallest of these axons was just visible along the most medial aspect of the hemi-
connectives (dotted axons in Fig. 6b,c). This pair of axons could not be traced within any
Figure 4: Photographs showing innervation of the salivary glands. One of the salivary duct neurons (arrowheads in (a) enter the salivary glands and produces many branches (b) which innervate the acini (straight arrows). A fine network of fibres can be seen over an acinus (c - curved arrows) which appear to arise from one of the sub-branches (straight arrow). Note also the varicose nature at many positions along the fine axon branches over the acinus. (d) Fine TH-positive processes (curved arrows) also extend over and appear to innervate the salivary gland reservoirs (SR). SD, salivary duct; Scale bar: 110μm (b,c,d); 200μm (a).
Figure 5: Photographs of the ventral thoracic ganglia (a, b, c) and a representative abdominal ganglion (d) of an immature animal (d). In the prothoracic ganglion note the intensely staining somata of the anterior median (AMN) and posterior median neurons (PMN). The axons of the AMNs cross-over to the contralateral side of the ganglion (see Fig. 6a), although they do not appear to project to the periphery. Other cells in the posterior ganglion stained less intensely but were always present (arrows and arrowheads). A single, lightly staining pair of cells in the mesothoracic ganglion (arrows in b) and a cluster of 3 bilaterally symmetrical cells in the metathoracic ganglion (arrows in c, left side only) were always observed. The most posterior cells in the clusters of the metathoracic ganglion were situated more dorsally and usually stained with less intensity. The first to fifth abdominal ganglia (d) contained a single bilateral pair of cells (LN). Two interneurons can also be seen coursing through the ganglion (arrows). Scale bar: 110μm (d); 200μm (a,b,c).
of the ganglia. Some axons of larger diameter often travelled close together, probably in a tract, which sometimes made identification of individual axons more difficult. Larger diameter axons could often be traced passing directly through a ganglion with little, if any, deviation (Fig. 6c). No branches from these axons were observed within the ganglia. There were no axons immunopositive for TH-like material in any nerve root of T1, T2 and T3.

**Unfused Abdominal Ganglia**

The first to fifth abdominal ganglia (A1 to A5) contained a single, bilaterally symmetrical pair of neurons which stained positive for TH-like material (LN, Fig. 7a). The axons of these cells did not stain well enough to determine a possible target. Intersegmental interneurons that stained for TH-like material passed through each abdominal ganglion and no branches from these interneurons were observed in the ganglia, nerve roots, or anywhere along the connectives.

**Terminal Abdominal Ganglion (A6)**

Three bilaterally symmetrical lateral somata (LN) were always immunopositive for TH-like material (Fig. 7b and 8d), with axons that were traced into the neuropile region. Several prominent axon tracts were observed in the interganglionic connectives just anterior to A6 (Fig. 7b; Fig. 8a). These axons branch and appear to form an elaborate network of arborizations that are distributed both laterally and in the medial posterior (ventral) part of the ganglion (dotted elliptical area, Fig. 7b; Fig. 8b,c). Although the general areas of dendritic networks were evident, the details of the fine branch patterns could not be compared amongst individual preparations. No axons staining for TH-like material were observed in any of the nerve roots associated with A6.

**Treated Groups**

Removing food from animals for a period of 24h to 1 week prior to experimentation did not result in improved staining for TH-like material. Prior injection of pargyline alone (25μg) usually resulted in an increased intensity of staining of TH-like material compared
Camera lucida drawings of the thoracic ganglia showing cells which stained positively for TH-like material. All cells are ventral except the most posterior pair of cells in the cluster of T3. (The outlines of the ganglia have been idealized.) Scale bar: ca. 500μm. (a) Prothoracic ganglion (T1). The anterior median neurons (AMN - filled cells) and the posterior median neurons (PMN) were the most intensely staining cells. The lateral neuron pair (LN) and median cell cluster (MCl) stained less intensely but were always present. (b) Mesothoracic ganglion (T2). Only a single pair of cells (PLN) stained for TH-like material in the mesothoracic ganglion. They were always present but occasionally difficult to locate because of their lightly staining nature. Three pair of prominent axons (INs) could usually be seen entering the ganglion from the interganglionic connectives. Some of these axons could occasionally be seen traversing the ganglion, although branches from these axons within the ganglia were not observed. Also, a pair of very fine axons (indicated by the dashed lines) was always found close to the most median aspect of the hemic connectives. (c) Metathoracic ganglion (T3). A group of three bilaterally paired cells (PLCl) were found in the posterior region of the metathoracic ganglion. Axons from these cells could not be traced with certainty, although at least one pair may send axons into the posterior interganglionic connectives. Two of the four pair of interneurons (INs) which traversed this ganglion are also shown.
to parallel-run controls. Most notable were the fine arborizations seen in the terminal abdominal ganglion (Fig. 8b,c) and the fine axons over the acini of the salivary glands (not shown). However, it should be noted that occasionally preparations from pargyline injected animals did not show a staining clarity or intensity different from controls. Higher doses of pargyline alone or pargyline followed by injection of GBL did not appear to improve staining of TH-like material.

Staining of tissue for TH-like material was not noticeably improved by the whole animal injection of reserpine 24h prior to fixation. Staining intensity of previously identified cells was the same or was reduced by forskolin injection 24h prior to fixation. The SN1 neurons of the SOG, which are normally the most intensely staining cells in the nervous system, were only lightly stained in forskolin injected animals (not shown). Areas of arborizations in the terminal abdominal ganglion were not visible and staining of axons over the salivary glands appeared reduced. Other areas were also more lightly stained than that seen in controls run in parallel.

Dopamine and Serotonin Content of Salivary Glands

Using HPLC coupled to an electrochemical detector, the dopamine content of the salivary gland was determined to be $16.2 \pm 1.8$ pmol/mg of protein and the serotonin content was $25.5 \pm 1.8$ pmol/mg protein (mean ± standard error of 8 determinations). Dopamine and serotonin content of the nervous system have been previously documented (Pitman, 1985).

Discussion

Immunohistochemical methods rely on the ability of antibodies to recognize and bind to sites on specific target molecules and as such the degree to which homologous target molecules in different animal species are recognized may vary. Antibodies made against TH have been used to identify catecholaminergic neurons in vertebrates (Saland et al., 1988; Leviael et al., 1991) without major specificity constraints. Recently, these antibodies
Figure 7: Camera lucida sketches of the general pattern of staining for TH-like material in the first to fifth abdominal ganglion (inclusive, A1-A5)(a) and the terminal abdominal ganglion (b) of an immature cockroach. The adult staining pattern is identical. Scale bar: ca. 500μm. (a) Only a single bilateral pair of somata located along the posterior-lateral margin of A1-A5 stained for TH-like material (LN). An axon could not be traced from the cell body. At least four axons (INs) were seen entering and exiting the anterior and posterior regions of the ganglia from the interganglionic connectives. Some of these could be traced across the ganglia. (b) Camera lucida sketches of the terminal abdominal ganglion (A6). Three bilaterally symmetrical somata (LN) which stained for TH-like material were always observed along the lateral margins of A6. The axons of these cells could only be traced a very short distance toward the neuropile region. Interneurons (curved arrows) entered A6 and formed an area of heavy arborization, the limits of which are approximately delineated by the dashed line vertical ellipsoid. Individual arbors (not shown) were difficult to accurately map although general areas of arborization were more clear (also see Fig. 8).
have been used in invertebrates to identify catecholamine-containing cells (Budnik and White, 1988; Nyhof-Young and Orchard, 1990) with a large degree of success. Thus, it appears that the commercially available TH monoclonal antibody is able to recognize the insect-equivalent TH.

We have investigated the distribution of TH in the ventral nerve cord (VNC) and salivary glands of the cockroach, *Periplaneta americana* (L.). As has been found in locust (Orchard *et al.*, 1992), the salivary gland of the cockroach appears to be the only peripheral target which receives axons from the ventral nerve cord that stain positively for TH. The salivary neurons (SN1), whose somata are located in the anterior and ventral portion of the SOG, stain intensely for TH, indicating a probable catecholamine content. Additionally, their axons, which project to the salivary glands, also stain positively for TH-like material. Under conditions of Falck-Hillarp fluorescence, the same cells show a positive reaction in both cockroach and locust, and the presence of dopamine (locust SN1s) was established using a radioenzymic assay (Gifford *et al.*, 1991). In cockroach, the SN1s are by far the most intensely staining neurons and their axons are clearly identifiable in almost all preparations. This intense staining for TH reflects upon the important role the enzyme (and putative neurotransmitter dopamine) plays in its association with a peripheral target organ - the salivary gland. Also, this association is important because it appears that this is the only peripheral target of putative dopaminergic neurons. A role for TH in communication within the CNS is indicated by its presence in interneurons which extend from the head to terminal abdominal ganglion.

In addition to innervation by SN1 neurons, the salivary duct nerve of the cockroach contains another axon (SN2) of comparable diameter (3-4μm) and several axons of smaller diameter (Whitehead, 1971). Somata of the SN2 neurons are located in the posterior-ventral region of the SOG and do not stain positively for TH (and thus probably do not contain dopamine or noradrenaline). The somata of the small diameter axons in the salivary duct nerve are believed to be in the SOG although they have not been successfully identified. Their axons do not appear to contain TH-like material (by immunohistochemistry) or catecholamines, but have been suggested to contain serotonin (Davis, 1985; Gifford *et al.*, 1991). These small axons that accompany the SN1 and SN2
Figure 8: Photographs of the terminal abdominal ganglion (A6) from an animal injected with pargyline. Anterior is to the left. (a) Anterior A6 showing TH-like material in axons of the interganglionic connectives entering the ganglion (curved arrows). The small white arrows point to one axon in the pair of the fine median axons that stained for TH. The area of (b) and (c) is the same. The level of focus of (b) is more superficial than (c). In (b), note the dendritic fields (arrowheads) that appear to emanate from the interneuron (straight arrows). At a deeper level of focus (c) other arborizations can be seen. (d) The right side of A6 showing three bilaterally symmetrical somata along the lateral margin of the ganglion that stained for TH-like material (arrows). Scale bar: 110μm (b,c); 200μm (a,d).
axons in the salivary duct nerve of cockroach seem to be absent in locust. Also of note is that SN2 has been shown to contain serotonin in the locust, but not in the cockroach.

Gifford et al. (1991) speculated that in Periplaneta these cells may contain octopamine since this amine is associated with the salivary glands of Nauphaeta cinerea. However, cockroach salivary glands also receive innervation from the stomatogastric nervous system (Whitehead, 1971), and it is plausible that the octopamine found associated with Nauphaeta salivary glands (Mitchell and Williams, 1981) arose from the stomatogastric innervation.

The role of dopamine as a putative neurotransmitter in salivary tissue has been firmly established. Dopamine has been shown to increase cyclic-AMP production, elicit fluid secretion, and induce hyperpolarization in salivary gland tissue (Bowser-Riley and House, 1976; Smith and House, 1977; Grewe and Kebabian, 1982). We have confirmed the presence of both dopamine and serotonin in Periplaneta salivary glands using HPLC and found the content to be comparable, although slightly less, than that found in locust (Ali et al., 1993).

Dopamine is also involved in the control of salivation in other insect species. For example, it has been found associated with the salivary glands of the hawkmoth, Manduca sexta, where it may play a role in modulating salivary secretion, as opposed to production, since only the fluid secreting regions are innervated (Robertson, 1974).

Other aspects of TH staining in the nervous system of cockroach exhibit similarities and differences with TH immunoreactive neurons in the locust. In Periplaneta and Locusta there is a pair of posterior median neurons (PMN, see Fig. 2) in the SOG which stain intensely, as well as pairs of cells in the other thoracic ganglia. The most striking difference between Locusta (Orchard et al., 1992) and cockroach occurs in the prothoracic ganglion.

Here, there are clear differences in numbers and position of TH-positive cells. For example, in cockroach, the axons of the anterior median neurons (AMN) cross over to the contralateral side of the ganglion. There does not appear to be a homologous pair in the locust prothoracic ganglion (Orchard et al., 1992), although they present evidence of an
anterior median lateral pair which send axons into the posterior ipsilateral connectives. Also, in the prothoracic ganglion, a median cluster (MCI) is present in cockroach but absent in Locusta (Orchard et al., 1992) and in Schistocerca using a dopamine specific antibody (Watson, 1992). The posterior medial neurons of Locusta (labelled "MN" in Orchard et al., 1992), and the PMN of cockroach, appear to correspond to the posterior medial prothoracic neurons identified in Schistocerca (using a dopamine specific antibody - Watson, 1992). It is interesting to note that in general, Watson (1992) found fewer cells staining positively for a dopamine-like substance in Schistocerca than are found staining positively for either catecholamines in Locusta (Villemaringe et al., 1981) and cockroach (Fleming and Pitman, 1983), or TH in Locusta (Orchard et al., 1992) and cockroach (this study).

Another notable similarity between Periplaneta and Locusta is that each unfused abdominal ganglion contains a single, bilaterally symmetrical lateral neuron. The terminal ganglion in the two animals are similar, although there are only three pairs of TH positive cells in Periplaneta (compared to 10-12 pair in Locusta (Orchard et al., 1992)). Interestingly, Falck-Hillarp fluorescence of A6 in Periplaneta did not reveal any fluorescent cell bodies (Dymond and Evans, 1979), but only the presence of a neuropile region. However, a later study (Baker and Pitman, 1989) using the glyoxylic acid method depicted three lateral pairs of somata (which is similar to the present study). The lower sensitivity of the Falck-Hillarp technique used by Dymond and Evans (1979) may be responsible for this discrepancy (Klemm, 1980), especially since these 3 pairs of cells in the terminal ganglion stained with only average relative intensity in our preparations. However, evidence of catecholamines in the neuropile region (Dymond and Evans, 1979) supports our finding of a rich supply of arborizations which stained for TH-like material.

The blood sucking bug, Rhodnius prolixus, has been intensely studied for the presence of catecholamines and TH in the brain (Flanagan, 1984; Nyhof-Young and Orchard, 1990) and VNC (Flanagan, 1986; Orchard, 1990). These studies have shown that there exists a generous distribution of both catecholamine- and TH-containing neurons in the brain and VNC of Rhodnius, and without exception they all appear to be interneurons. This finding
is similar to that which we describe for *Periplaneta*, where there is an absence of immunochemical staining of TH-like material in axons to peripheral targets (except for the salivary gland). A feature in *Rhodnius* which differs from that in *Periplaneta* (and *Locusta*) is the occurrence of ventral unpaired median neurons in each of the SOG, prothoracic ganglion and mesothoracic ganglionic mass (Flanagan, 1986; Orchard, 1990). *Drosophila* VNCs also possess unpaired median neurons (Budnik and White, 1988) with contents reactive to catecholamine and TH antibodies, and again, in this animal, these neurons appear to be interneurons with no peripheral projections. These differences are likely to be due to phylogenetic differences rather than the staining procedure.

A particular concern of this study was to more firmly establish the location of putative dopaminergic neurons in the VNC of the cockroach by comparing our results with those previously reported (Baker and Pitman, 1989) using the glyoxylic acid method (which identifies catecholamine containing neurons). Overall, there appears to be close correspondence between cells identified using the two techniques, with a few notable exceptions. For example, in the SOG, each anterior cluster (AC1 - Fig. 2) which accompanies the SN1 neurons contains 4 small cell bodies, whereas only 3 were previously reported (Baker and Pitman, 1989). As we have noted, this difference may be the result of one pair of cells occasionally being obscured by the others. Also noted is the absence of the lateral neuron pair (our LNP in the SOG - Fig. 2), the presence of a strongly fluorescent dorsal unpaired median cell body (also in the SOG), and an extra posterior lateral neuron pair in each of the thoracic ganglia by the glyoxylic acid method (Baker and Pitman, 1989). Another possibility for the presence of these cells by the glyoxylic acid method but their absence by the TH-immunohistochemical method is that they may contain a catecholamine synthesized by a pathway different from the putative primary synthetic path for catecholamines that involves TH. Thus, it is possible that these neurons contain a catecholamine (probably dopamine or noradrenalin) which could be formed using a minor synthetic path for dopamine or noradrenalin (tyrosine-tyramine-dopamine-noradrenalin or tyrosine-tyramine-octopamine-noradrenalin)(Cooper *et al.*, 1991; Owen and Bouquillon, 1992). In support of this possibility is evidence which indicates
that both octopamine and noradrenalin are present in the cockroach nervous system, although noradrenalin is present in very low quantities (Dymond and Evans, 1979; Pitman, 1985). More recently, an antibody against octopamine has revealed octopamine-like immunoreactive material in cells in the ventral nerve cord of the cockroach (Eckert et al., 1992). These cells appear to be distinct from those stained for TH described in this paper.

Injection of pargyline prior to dissection and fixation was the only drug treatment which seemed to noticeably improve staining of TH-like material in tissues. It was reported that prior treatment with pargyline and gamma-butyrolactone (GBL) enhanced immunoreactivity for TH-like material in nigrostriatal axons of mice by increasing the number of TH binding sites in the fixed tissue (Haykal-Coates et al., 1991) rather than by increasing the concentration of TH present in cells. Injection of pargyline into cockroaches (Omar et al., 1982) caused a small decrease in the dopamine content of cerebral ganglia. This corresponds with our results that indicate pargyline injection enhanced staining. This could be due to an increase in the titre of TH caused by a pargyline-induced decrease in dopamine. Differences in enhancement compared to mice (Haykal-Coates et al., 1991) may be due to a number of factors including species, metabolic rate, and temperature.

Although reserpine has been reported to increase TH activity (Sorimachi, 1975), mRNA concentration (Pasinetti et al., 1990) and TH enzyme protein (Reis et al., 1974), it was not effective in enhancing staining of TH-like material in cockroach nervous system or salivary glands. Baker and Pitman (1989) found reserpine caused a decrease in staining for catecholamines in axons and cell bodies using the glyoxylic acid technique. We used reserpine in a similar fashion (i.e., to decrease stores of catecholamines) in an attempt to increase enzyme activity as the cells try to recover from the deficit. Unfortunately, under the protocol described here, the results did not support this hypothesis. Similarly, forskolin, which has been shown to induce mRNA production in the substantia nigra of rats (Leviel et al., 1991), was also ineffective or in some preparations actually appeared to decrease staining intensity. Forskolin is also known to activate adenylate cyclase and to elevate cyclic-AMP. However, why forskolin (and reserpine) did not enhance TH staining
in cockroach remains to be determined.

In conclusion, we have shown that the distribution of immunochemical staining of TH-like material in the SOG and salivary glands support previous data on catecholaminergic neurons and innervation, and moreover, we outline previously undescribed neurons in the ventral nerve cord of *Periplaneta*. These neurons contain a TH-like material and are probably dopaminergic neurons. It is suggested that they likely function primarily as interneurons for communication and coordination of activity in neural circuits within the central nervous system. Their interaction with, for example, motor neurons, and their role in the generation or maintenance of activity will be investigated, and will lead to a better understanding of the role of catecholamines in the insect nervous system.

References


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VIII. Neuroanatomy and neurochemistry: implications for insect phylogeny.

Abstract

Physiologists and developmental biologists routinely collect data that when analysed from a phylogenetic perspective may improve our understanding of the evolutionary relationships of many enigmatic groups of organisms. Immunohistochemical studies of the innervation of the salivary glands provide data that clarify the evolutionary relationships of the lower Neoptera, the major unresolved issue in insect phylogeny of the orders of insects. Phasmatodea and Orthoptera are regarded as sister taxa based on the dorsal position in the subesophageal ganglion of the cell body of salivary nerve 1 and presence of serotonin in salivary nerve 2.
Introduction

It is remarkable that we do not have a better understanding of the evolutionary radiation that resulted in at least 80% of terrestrial species diversity. This is due not only to the absence of a robust phylogeny for the orders of insects but also a lack of appreciation of the importance of phylogenetic information in formulating and testing evolutionary scenarios; the current debate over the evolution of flight in insects is but one example (Marden and Kramer, 1994a, 1994b). Mitigating factors include the 390 million year history of insects with a paucity of fossils in the first 60 million years when diversification began (Labandeira and Sepkoski, 1993). Ordinal diversity peaked in the late Palaeozoic and early Mesozoic and almost one-third of these orders are extinct today (Kukalová-Peck 1991). It is true that much of the morphological evidence of kinship has been "diluted through eons of unique evolution" (Wheeler, 1989; Carmean et al., 1992), but the comparative anatomy and physiology of insects has not been fully mined for characters. In recent years, DNA sequence data has been brought to bear on the contentious issues of insect phylogeny, but with little resolution (Wheeler, 1989; Carmean et al., 1992). It is now clear that what is required is a "total evidence" approach (Kluge, 1989; Wheeler et al., 1993; Huelsenbeck et al., 1996), one that does not focus on the source of characters but extracts and summarizes phylogenetically informative data simultaneously from all sources of characters, e.g., morphological, behavioral, electrophoretic, chromosomal, and gene sequence data. Neurological data have provided insights into the evolution of flight in insects (Robertson et al., 1982) and mammals (Pettigrew, 1986; Simmons, 1994), but cellular and histochemical data have not been used to address the outstanding questions of insect phylogeny.

The "lower Neoptera" all have chewing mouthparts and incomplete metamorphosis -- and includes such well known insects as grasshoppers, crickets, walking sticks, earwigs, cockroaches, mantids, and termites. The monophyly of the mantids plus cockroaches plus termites "is perhaps the only major advance in this century of the high-rank interrelationships of lower neopteran insects" (Kristensen, 1989) and is supported by both
morphological (Thorne and Carpenter, 1992), gland structure (Seelinger and Seelinger, 1983), and molecular data (Kambhampati, 1995). All recent treatments of insect phylogeny leave the relationships of the eight extant orders (Plecoptera, Dictyoptera, Grylloblattodea, Dermaptera, Orthoptera, Phasmatodea, Embioptera and Zoraptera) completely unresolved and there is even the suggestion that the Orthoptera may be paraphyletic with respect to the Phasmatodea (Kristensen, 1991a; 1991b). To date, only morphological data have addressed these issues. In this report we present neurochemical and neuroanatomical characters that help to resolve the evolutionary relationships within the lower Neoptera. In addition to their intrinsic value, and perhaps more importantly, the findings also demonstrate that there are levels of biological organization between gross morphology and gene sequences that can help elucidate genealogical relationships.

Materials and Methods

Odonata: Libellulidae, *Sympetrum obtrusum* (Hagen). Adults were collected near Toronto in early September, 1995. Nymphs of *Agnetina* sp. (Plecoptera: Perlidae) were collected at Duffin Creek, Ontario in March of 1996. Voucher specimens are deposited in the Royal Ontario Museum. Animals were returned to the laboratory on the day of capture, and immediately dissected to remove tissues for immunohistochemical staining and high performance liquid chromatography coupled with electrochemical detection (HPLC-EC). Neuroanatomical information was obtained from TH-like, serotonin-like and FMRFa-like immunohistochemistry on suboesophageal ganglia and salivary glands. For *Sympetrum*, tissues were dissected under physiological saline (153 mM, NaCl; 2.6 mM KCl; 1.8 mM, CaCl₂; 24 mM glucose; pH 6.8) and fixed in 2% paraformaldehyde in Millonig’s buffer for 1 h. *Agnetina* were split open along the midsection from the head to abdomen and flooded with fixative (2% paraformaldehyde in Millonig’s buffer) for 1 h. The preparations were then washed several times in phosphate-buffered saline and the SOG and salivary glands were removed for immunohistochemical staining or HPLC analysis. Tissues were washed several times in phosphate-buffered saline (PBS) (10 mM
phosphate buffer, pH 7.2 containing 0.9% NaCl) for 4-6 h, then incubated in 0.4% Triton X-100 in (PBS) for 1 h at room temperature. Preparations were then processed for either TH-like, serotonin-like or FMRFa-like immunoreactivity. For TH-like immunoreactivity, tissues were incubated for 48 h at 8°C in a mouse monoclonal antibody generated against TH (Incstar Corp., Stillwater, MN, U.S.A.) diluted 1:500 in PBS containing 0.4% Triton X-100, 2% normal goat serum and 2% bovine serum albumin. The tissues were washed for 6-8 h in PBS, then incubated for 24 h at 8°C in a 1:200 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin G (Jackson Immunoresearch Labs, West Grove, PA) in PBS containing 10% normal goat serum. Preparations were then washed in PBS for 24 h, mounted and viewed in 5% n-propyl gallate in glycerol, pH 7.3. Serotonin-like and FMRFa-like immunoreactivity of the SOG and salivary glands were investigated using similar techniques. The primary serotonin antiserum was a 1:1000 dilution of a rabbit anti-serotonin antiserum (Incstar Corp.) and the secondary antibody was a goat anti-rabbit immunoglobulin G labelled with FITC. The primary FMRFa antiserum was a rabbit anti-FMRFa antiserum (Incstar Corp.) and the secondary antibody was the same as that used for serotonin-like immunoreactivity.

Parsimony analysis was carried out using an exhaustive search algorithm, D.L. Swofford, 

Results

There is a single shortest tree when Hemiptera alone is used as outgroup (length = 11, CI = .73); and three equally parsimonious trees when Odonata alone is used as outgroup (length = 10, CI = .80) and the strict consensus tree supports the sister group relationship of Orthoptera and Phasmatodea. The character polarity and optimization in Figure 2 is based on successive outgroups (Hemiptera, then Odonata) based on well substantiated
Figure 1. Data matrix and most parsimonious cladogram of neuroanatomical and neurochemical characters in lower Neoptera; Hemiptera primary outgroup, equivocal characters (5 and 7) polarized using Odonata. Shared derived characters indicated by bolded capital letters for the ingroup taxa, an asterisk (*) indicates reversals and a slash (') indicates independent derived states.
<table>
<thead>
<tr>
<th>CHARACTERS</th>
<th>CHARACTER STATES</th>
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<tbody>
<tr>
<td>1. Salivary nerve from stomatogastric system</td>
<td>Odon Plec Phasm Orthop Dictyp Hemip</td>
</tr>
<tr>
<td></td>
<td>? ? NO NO yes yes</td>
</tr>
<tr>
<td>2. Salivary reservoir; presence or position</td>
<td>proximal ABSENT ABSENT ABSENT distal distal</td>
</tr>
<tr>
<td>3. Salivary duct innervated</td>
<td>no NO yes NO NO yes</td>
</tr>
<tr>
<td>4. SNS associated with salivary nerve</td>
<td>yes NO NO yes yes</td>
</tr>
<tr>
<td>5. SN1 cell body in suboesophageal ganglion</td>
<td>ventral ventral DORSAL DORSAL ventral ?</td>
</tr>
<tr>
<td>6. Serotonin present in SN2</td>
<td>? no YES YES no no</td>
</tr>
<tr>
<td>7. FMRFa innervation of salivary gland</td>
<td>absent TMN absent TMN absent STS</td>
</tr>
</tbody>
</table>

Length = 12
CI = 0.75
aspects of insect phylogeny (Kristensen, 1989; Seelinger and Seelinger, 1983). The resultant cladogram has three unique and unreversed synapomorphies (#1, 5, 6) for the clade containing Orthoptera and Phasmatodea. Characters 5, 6 are shown in Figure 1; character 1 is ambiguous in the combined analysis due to missing values.

Character analysis: 1. Salivary nerve from the stomatogastric system: yes or no. The salivary glands in *Periplaneta* and *Rhodnius* are innervated via a nerve from the stomatogastric system (Davis 1985; Tsang and Orchard, 1991). Innervation appears to be absent in *Locusta* and *Carausius* and (this character could not be evaluated) in the dragonfly and stonefly. 2. Salivary reservoir: distal, proximal or absent. A pair of large and well defined salivary reservoirs (one associated with each gland) is present in *Periplaneta* but absent in *Locusta* and *Rhodnius*. Each *Rhodnius* salivary gland contains an accessory gland, which is thought to be homologous with the salivary reservoir of other insect species (Baptist, 1941). 3. Salivary duct innervated: yes or no. The primary salivary ducts of *Carausius* are innervated by serotonergic and dopaminergic immunoreactive fibres along its length. The salivary glands of *Rhodnius* are also covered with a meshwork of serotonin-like and FMRFa-like immunoreactive fibres. This pattern of innervation is in contrast to *Locusta*, *Periplaneta* and the dragonfly and stonefly in which the salivary ducts do not appear to be innervated by serotonin, dopamine, or FMRFa. 4. Serotonergic neurohaemal system (SNS) associated with the salivary nerve: yes or no. The salivary glands of *Locusta* receive innervation from an extensive serotonergic neurohaemal network (SNS). An SNS is thought to be associated with the salivary glands of *Periplaneta* (Davis, 1985; 1987), *Rhodnius* (Orchard et al., 1988; Orchard, 1990; Tsang and Orchard, 19913) and the dragonfly but this pattern of innervation is apparently absent in the stonefly. 5. Position of the SN1 cell body within the SOG: dorsal or ventral (Fig. 2). SN1 is situated on the dorsal surface of the SOG in *Locusta* and *Carausius* whereas it is located on the ventral surface in *Periplaneta* and in the dragonfly and stonefly. 6. Serotonin present in the SN2: yes or no. SN2 in *Locusta* and *Carausius* contains serotonin (Ali and Orchard, 1996; Gifford et al., 1991; Ali et al., 1993) (Fig. 2) but the SN2 of *Periplaneta* does not appear to be serotonergic. The
Figure 2. Position of the salivary neurons within the suboesophageal ganglion of 3 orthopteroid insects, A) *Locusta migratoria*, B) *Carausius morosus*, C) *Periplaneta americana*. Filled circles represent cells that contain dopamine; stippled circles represent cells that contain serotonin; open circle represents an unknown phenotype; solid line represents cells on the dorsal surface; broken line represents cells on the ventral surface. SN1, salivary neuron 1; SN2, salivary neuron 2; SLNv, salivary nerve.
A

Locusta migratoria

B

Carausius morosus

C

Periplaneta americana
absence of serotonin-like immunoreactivity in the salivary nerve of the stonefly suggests the absence of serotonin. 7. FMRFa innervation of salivary glands: absent or produced by transverse median nerves (TMN) or from the stomatogastric nervous system (STS). Positive FMRFa-like immunoreactivity of the salivary glands of Locusta, Rhodnius, and the stonefly indicates the presence of these peptides. However, these peptides originate from different sources (Fuse et al., 1996; Myers and Evans, 1985; Tsang and Orchard, 1991). FMRFa-related peptides have not been found associated with the salivary glands of Carausius, Periplaneta and the dragonfly.

Discussion

The discovery of identifiable aminoergic neurons is an important step towards a description of the physiological relevance of amines within the insect nervous system. It was in this context that immunohistochemical studies were conducted on three of the species commonly used in insect physiology laboratories: Carausius morosus, Locusta migratoria and Periplaneta americana. Immunohistochemical staining of the ventral nerve cord of the Indian walkingstick with an antibody against tyrosine hydroxylase suggested the presence of a pair of dopaminergic salivary neurons (SN1) within the suboesophageal ganglion. Cobalt backfilling of the salivary nerve revealed a second salivary neuron pair (SN2), which in subsequent experiments were found to stain positively with an anti-serotonin antiserum (Ali and Orchard, 1996). Comparisons with published information on the migratory locust and American cockroach revealed some striking similarities in the innervation of the salivary glands of these three species. Briefly, the salivary nerves of each species contain two main axons whose cell bodies (SN1 and SN2) are located within the suboesophageal ganglion (Fig. 2) (Ali and Orchard, 1996; Altman and Kien, 1979; Gifford et al., 1991). The SN1 are dopaminergic and contralateral to the salivary nerve root and the SN2 are ipsilateral to the nerve root (Ali and Orchard, 1996; Altman and Kien, 1979; Gifford et al., 1991; Ali et al., 1993); the
SN2 are serotonergic only in the locust and walkingstick (Ali and Orchard, 1996; Gifford et al., 1991; Ali et al., 1993). Furthermore, the locust and cockroach have a serotonergic neurohaemal nervous system associated with the salivary nerve (Bräunig 1989; 1988).

The similarities and differences in the innervation of the salivary glands of these three species suggested the possibility of using these data to address the issue of the phylogenetic relationships among these orders. A prerequisite for a phylogenetic analysis is outgroup comparisons to determine character state polarity. Comparative data were obtained for two outgroup taxa, a dragonfly, which is basal to the lower Neoptera, and a true bug, which is more derived (Kristensen 1991a; 1991b), and for a stonefly, an ingroup taxon that is a "potentially basal group in Neoptera" (Will, 1995). These data resulted in seven informative characters: one gross morphological feature at the tissue level (#2) and six neurochemical or neuroanatomical characters (Fig. 1). The data matrix was subjected to parsimony analysis with both Hemiptera and Odonata serving as outgroups and with both of these taxa included in a simultaneous analysis. These data were also added to a comprehensive morphological database for 33 taxa and 251 morphological characters (Whiting et al., In Press) and in a parsimony analysis of these combined data the neuroanatomical and neurochemical characters provide two unique and unreversed synapomorphies for the clade containing Orthoptera and Phasmatodea.

These results suggest that important comparative information can be extracted from neuroanatomical and neurochemical studies and that comparative data collected by physiologists and developmental biologists can and should be incorporated into a comprehensive approach to phylogenetic reconstruction. A closer collaboration between biologists offers the best prospect of improving our understanding of the evolutionary relationships of many enigmatic groups of organisms.

References


As we strive to further our understanding of neuroscience we have learned to study those systems which best suit our needs. To this end, invertebrate preparations have been favoured by biologists. Perhaps the best known neurophysiological preparation is the squid giant axon whose extraordinarily large size offered (and gave) the best hope of studying the basis of the action potential and the mechanism whereby information is passed along nerve fibres (Hodgkin and Huxley, 1939; 1952). Since then several invertebrate preparations have offered numerous insights into the fundamentals of neurophysiology. The crayfish neuromuscular junction has been extensively studied in order to determine the events underlying neuromuscular transmission (Mercier et al., 1990). In a similar fashion, developmental neurobiology has flourished with the use of molluscan embryos as preparations to study neuronal growth and embryogenesis (Cohan et al., 1987; McCobb et al., 1988). In addition, the suitability of Drosophila in genetic studies also leads to advances in developmental biology (Burke and Basler, 1996; Riesgo-Escovar et al., 1996;). Thus, invertebrate preparations have played a central and invaluable role in the advancement of neuroscience. There are several reasons for this. Invertebrates have a relatively simple nervous system in comparison to vertebrates. This is of course a matter of degrees, since the central nervous system of an insect may be composed of several ganglia and several hundred thousand nerve cells. Invertebrates tend to have large nerve cell bodies positioned around the periphery of the ganglia while the ganglionic center is reserved for axonal tracts and integrative neuropile regions. This structural division usually allows for easy accessibility to the neuronal somata. Another important feature of the invertebrate nervous system is that positioning of the somata does not change in any appreciable way, thus, one can move from animal to animal within a given species, and locate the same cell in virtually every instance. The large size of some cell bodies (50-1000 μm) is advantageous for intracellular studies. In addition, one can usually acquire large numbers of experimental animals in a relatively short period of time. Finally, invertebrates possess chemical substances that are also
found in animals representative of virtually every phylum in the animal kingdom. If one wishes to study the interactions, modulation and the effects of neuroactive substances, the central nervous system of invertebrates offer well-defined, easily accessible systems to do so.

The present study on the aminergic innervation of the salivary glands of the African migratory locust, *Locusta migratoria*, was undertaken because of the preparations usefulness for investigating amine-amine and amine-peptide interactions. Given the doubts in the literature regarding the specific aminergic content of salivary neurons, SN1 and SN2, this study was carried out to properly characterise the system. This was done in order to more fully understand aspects of the innervation of the salivary glands, and to lay the foundation, if only in part, for future research on this preparation. During the course of this study, the innervation of locust salivary glands has been revealed to be more elaborate and complicated than was initially assumed. Short discussions have appeared in each chapter in the body of this work. However, a more consolidated and overall view will be presented in this section. In addition, I have tried to address issues that were not addressed in the individual discussions.

In Chapter II, evidence was presented for the dopaminergic and serotonergic innervation of the salivary glands via SN1 and SN2 respectively. The SN1 stain positively for tyrosine hydroxylase and the SN2 stain positively for serotonin. Positive TH-like immunoreactivity of SN1 strongly suggests a catecholaminergic presence, which of course includes the presence of noradrenaline. Insects tend to contain much more dopamine than noradrenaline (Evans 1980), and it is probable that the SN1 contain dopamine rather than noradrenaline. We did not specifically test for the presence of noradrenaline, but we do know that dopamine is present in SN1 as indicated by Falck-Hillarp fluorescence and radioenzymatic assay (Gifford *et al.*, 1991). At the level of the salivary glands we know that dopamine is capable of inducing elevations of cyclic AMP while noradrenaline has little effect on cyclic AMP levels (Ali and Orchard, 1994). In addition, noradrenaline appears to have a potency of 0.1% that of dopamine in inducing salivation in isolated salivary gland bioassays (Baines and Tyrer, 1989). Although noradrenaline was suggested to exist in the salivary glands (Baines *et al.* 1989), its presence was not confirmed in
either pair of SN1 or SN2 (Baines et al., 1989). Does noradrenaline play a role in the glands other than salivation, or does it have any role at all? This remains to be seen, but the evidence to date suggests that noradrenaline may not play a role in salivation. Nevertheless, the presence of dopamine within SN1 is well established. This amine is certainly capable of inducing salivation (Baines and Tyrer, 1989), and elevates cyclic AMP levels in a dose-dependent manner in the salivary glands (Ali et al., 1993). Is cyclic AMP directly involved in the salivation response to exogenously applied dopamine? We do not know. At first thought it seems reasonable to assume that it is on the basis of studies performed on cockroach and blowfly salivary glands. Cyclic AMP is capable of inducing salivation in the cockroach, *Nauphoeta cinerea* (Grewe and Kebabian, 1982) and in the blowfly, *Calliphora erythrocephala* (Berridge, 1970). However, this has not yet been tested in locusts. The precise mode of action of cyclic AMP in salivation is still largely unknown.

In order to produce their effects, serotonin and dopamine must bind to receptors in the salivary glands to initiate the transduction mechanisms which mediate fluid secretion. A partial characterization of receptors that bind to dopamine and serotonin and that are coupled to adenylate cyclase was performed. This study is given in the second chapter where a variety of receptor antagonists and agonists (for each amine) were tested for their ability to either block or mimic the effects of the amine. The results suggest the presence of receptors that have similarities with vertebrate dopaminergic, D1-like and serotonergic, 5-HT2-like receptors. I have referred to this study as a partial characterization for the following reasons: first, since we measure levels of cyclic AMP as our response, we are unaware of changes in the intracellular levels of other second messengers, or of changes in the conformation of ion channels which may be affected by the amine. Secondly, we used several different classes of drugs which we felt were adequate for the study; however, the use of a wider variety of drugs would theoretically allow for a fuller characterization of the receptors. A study of such enormity, however would not be feasible. Thirdly, full characterization of receptors entails 3 kinds of studies of which only 2 were performed: sequencing data should be available which gives the primary amino acid structure of the receptor so that it can be compared with known sequences; operational studies where one
tests the potencies of antagonists to block the effect of the natural ligands, and of agonists to induce the effect of the ligand; transductional studies which examine the transduction mechanism which mediates the effect of the natural ligand. Clearly, we are lacking the sequencing data for the dopaminergic and serotonergic receptors, but the operational and transductional studies suggest the presence of D₁-like and 5-HT₂-like receptors that are positively coupled to adenylate cyclase.

The elevation of cyclic AMP in the glands can be mimicked by electrical stimulation of the salivary nerves with extracellular electrodes (Ali and Orchard, 1994). Partial block of the neurally-induced elevation of cyclic AMP by the dopaminergic antagonist, SCH-23390, and the serotonergic antagonist, spiperone, suggests that stimulation of the salivary nerve releases dopamine and serotonin from the terminals of SN1 and SN2. Interestingly, stimulation of the salivary nerve still leads to elevated cyclic AMP levels of glands in the presence of both aminergic antagonists. We first introduced the hypothesis that this remaining elevation could still be due to serotonin binding, since no serotonergic antagonist was completely effective, or that stimulation of the salivary nerve led to the release of other substances that are also capable of elevating cyclic AMP levels (Ali and Orchard, 1994). In addressing the first hypothesis we may assume that SCH-23390 is capable of blocking the effects of dopamine by 100%, thus the elevated cyclic AMP levels in preparations stimulated in the presence of SCH-23390 is likely due to the effects of serotonin, and is roughly equivalent to 20 pmol/mg of protein of cAMP. The serotonin dose-response curve indicates that exogenously applied serotonin at 5x10⁻⁸ M induces an elevation of cyclic AMP of approximately 30 pmol/mg of protein. This concentration of serotonin is sufficiently low such that spiperone at 10⁻⁵ M is virtually 100% effective at blocking the effects of serotonin. Thus, the effects of the serotonin released in response to stimulation of the salivary nerve should theoretically be blocked by the antagonist, spiperone. With regard to the second hypothesis, a recent study identified, isolated and sequenced a 15 amino acid peptide (NH₂-EVGDLFKEWLQGNMN-COOH) from the salivary glands of Locusta, which is capable of stimulating salivation and of elevating cyclic AMP levels in the salivary glands in a dose-dependent manner (Veelaert et al., 1995). It is therefore possible that stimulation of the salivary nerve leads to the release of
the peptide whose effects cannot be blocked by dopaminergic and serotonergic antagonists.

Several lines of evidence suggest that dopamine and serotonin are neurotransmitters in SN1 and SN2 respectively. For instance, dopamine is present in SN1 while serotonin is present in SN2. Exogenously applied dopamine and serotonin induce salivation and induce elevations of cyclic AMP levels in the salivary glands. Electrical stimulation of the salivary nerve leads to elevations of cyclic AMP levels that can be partially inhibited by dopaminergic and serotonergic receptor antagonists. However, are dopamine and serotonin true neurotransmitters in this system? The answer to this question is not as straightforward as one may initially assume. The fulfillment of neurotransmitter criteria is generally difficult to assess for several reasons and the experiments performed in Chapter 3 were designed to address the issue of neurotransmitter criteria for serotonin and dopamine. Extensive attention has been paid to these criteria but additional comments are warranted. As stated by Werman (1966), the synaptic criteria were "originally meant to prevent the intrusion of careless work and the neglect of scientific methodology". As such, several of the criteria need closer inspection. The criteria of the presence of an inactivating enzyme in the region of the synaptic cleft is perhaps the most obvious for examination since diffusion alone may account for a dramatic decrease in ligand concentration at receptor sites. Also, the presence of uptake transporters, particularly for the biogenic amines and amino acid transmitters, nullifies the need for inactivating enzymes at or close to the synapse. Therefore, in chapter 3 we have referred to this criterion as the existence of a mechanism for removal of the chemical rather than the presence of an inactivating enzyme. The criterion of collectibility of the transmitter is usually difficult to assess since relatively large quantities must be released in amounts which overwhelm the removal mechanism such that excess transmitter can be collected. This was achieved with the salivary glands because of the ability to measure concentrations of radiolabelled amine in the fmol range, but more importantly, because it appears that actual synaptic sites are rare (if not altogether absent); however, release terminals are abundant (Peters et al., 1987). The absence of a synaptic barrier to diffusion ensures that a greater quantity of transmitter will diffuse into the surrounding medium where there is more likelihood of collection. Certainly, the most important criteria are the
criteria of identical actions and of pharmacological identity; we believe that these have been sufficiently fulfilled. Briefly, evidence exists for the sialagogic effects of serotonin and dopamine on the salivary glands, and of salivation due to stimulation of the salivary nerve (Baines et al., 1989). Also, exogenously applied serotonin and dopamine elevate cyclic AMP levels in the glands while stimulation of the salivary nerve does the same (Ali et al., 1993; Ali and Orchard, 1994). More importantly, drugs which modify the action of applied dopamine and serotonin on cyclic AMP levels in the glands also modify, in a similar manner, the effect of electrical stimulation of the salivary nerve on cyclic AMP levels in the glands (Ali and Orchard, 1994). Thus, serotonin and dopamine may be considered true neurotransmitters in the salivary glands of the locust.

What of the activity of the salivary neurons? Perhaps the most notable results from this section are the recruitment of SN1 activity via electrical stimulation of the maxillary nerve, and the activity induced in SN1 and SN2 by carbachol and nicotine. Why should stimulation of the maxillary nerve only lead to activity in SN1 and not SN2? This seems to be an additional line of evidence to suggest separate roles for dopamine and serotonin in this system. If, as postulated by Schactner and Bräunig (1995), dopamine induces the secretion of a watery saliva lacking protein, then the initial activity of SN1 may be to ensure that the mouthparts are properly lubricated prior to feeding. If the animal does not feed, then precious proteins and enzymes are not expended in vain.

Insects are known to use acetylcholine as a neurotransmitter in the central nervous system. Not surprisingly, cholinergic agonists have a stimulatory effect on SN1 and SN2. Nicotine in particular, is very effective, implying the presence of nicotinic-like receptors on neurons which impinge upon the pathway for salivary neuron activation. The neural circuitry involved in feeding and in the control of the salivary neurons is unknown. We suspect that the salivary neurons are inhibited when the animal is not feeding, although SN1 periodically loses this inhibition during interfeeding bouts (Schactner and Bräunig, 1995). Both neuron pairs become active prior to the actual chewing of food, and cease shortly after mandibular muscle activity has ceased (Schactner and Bräunig, 1995). Activity in the maxillary nerve seems to affect SN1 firing, and activity in the transverse-median nerves of the prothoracic and mesothoracic ganglia affect SN1 and SN2 firing.
We are not in a position to decipher the intricacies of the control of feeding; more information is needed to tackle this complicated issue.

Finally, a comparative study on the innervation of the salivary glands was performed between the locust, *Locusta migratoria*, the cockroach, *Periplaneta americana*, and the stick insect, *Carausius morosus*. This was done to investigate the possibility of the presence of aminergic salivary motorneurons in *Carausius* and *Periplaneta*, with the hope that we would be presented with an additional preparation to study aminergic innervation. In addition, it is likely that further studies on the salivary glands of these species may reveal common means for the control of peripheral systems and of stimulus secretion coupling mechanisms. An interesting outcome was the development of the neuroanatomical and neurochemical information as characters in a phylogenetic analysis of these groups within the Lower Neopteran orders of insects (Chapter 8). This study, performed in collaboration with Dr. D. Christopher Darling, an Entomologist and Phylogeneticist, suggests that neuroscientific data have the potential to play an important role in determining phylogeny. Previously, the exact phylogenetic relationship between the orders to which these three species belong was unknown. The fundamental characters which allow us to come to our conclusion are i) the dorsal position of the SN1 in *Locusta* and *Carausius* in contrast to its ventral position in *Periplaneta*, and ii) the presence of serotonin in SN2 in *Locusta* and *Carausius*, but not *Periplaneta*. Outgroup comparisons had to be performed in order to properly polarize the characters. Thus, the innervation of the salivary glands of a representative of a dragonfly species and a stonefly species was examined. This was done in concert with the compilation of data on the innervation of the salivary glands of *Rhodnius prolirus*. The major finding of the study, that locusts and stick insects are sister groups and are more closely related to each other than either is to cockroaches was suspected, but until now, unconfirmed. However, the primary importance of the study lies in its assessment of the use of neurochemical data as characters in phylogenetic analyses. To date, morphological, molecular and behavioural data have been the major sources of phylogenetic information, but here, we suggest that neurochemical data may also be of some importance. Studies have used neurological data
to examine aspects of evolution in arthropods and mammals (Breidbach et al., 1995; Robertson et al., 1982; Pettigrew 1986), but a proper, full phylogenetic analysis has not been performed.

What is the physiological significance of these characters? This question cannot be properly answered due to a lack of physiological data but tentative theories can be presented. The lack of serotonin in the SN2 is an interesting occurrence, particularly since both serotonin and dopamine influence salivation in this insect (Just and Walz, 1996). The serotonergic source is probably a neurohaemal serotonergic system whose somatic origins can be found in the SOG (Davis, 1985), although serotonergic oesophageal axons project to the salivary glands from the stomatogastric nervous system (Davis, 1985). The salivary glands of Carausius only receive serotonergic innervation from SN2 while the glands of Locusta receive innervation from SN2 and a serotonergic neurohaemal system (Bräunig, 1987, 1988). Did cockroaches lose the serotonin from the SN2, already having 2 separate sources of salivary innervation negating the need for a third, or did Locusta and Carausius gain a serotonergic phenotype? The phylogenetic analysis is done from the perspective of Locusta and Carausius gaining a serotonergic SN2, but losing the stomatogastric innervation, which may have been serotonergic. In the final analysis, serotonin remains associated with the salivary glands of all 3 species, validating its importance in salivation.

A recurring question of this study deals with the presence of both serotonin and dopamine associated with the salivary glands of Locusta. In other words, why are both amines present if they do the same thing? At first glance these amines may appear to perform identical functions: that is, inducing salivation, with concurrent increases in cyclic AMP levels in the glands; however, these results require closer inspection. As mentioned in Chapter 2, the salivary glands of the desert locust, Schistocerca gregaria are composed of several cell types, including zymogenic and parietal cells (Kendall 1969) which perform slightly different functions. We postulated at the time that the receptors for serotonin may be associated with one cell type, while the dopaminergic receptors may be associated with the other (Ali and Orchard, 1994). Since then, new information on cockroach salivation
has come to light, which may strengthen our supposition. Just and Walz (1996) found that the saliva secreted by the salivary glands of *Periplaneta americana* in response to serotonin had a high protein content, while the saliva secreted in response to dopamine consisted of water and ions and lacked protein. Given license to speculate, we may be tempted to envision a similar situation in *Locusta*. Such a model is shown in Figure 1, where dopamine and serotonin are released from the terminals of the SN1 and SN2 and act on the zymogenic and parietal cells respectively. Dopamine elevates cyclic AMP and possibly Ca\(^{2+}\), which results in ion and water flow into the lumen of the acini. Serotonin elevates cyclic AMP and possibly Ca\(^{2+}\), which mediate the release of salivary enzymes into the lumen of the acini.

Other lines of evidence also suggest separate roles for serotonin and dopamine. For instance, the SN1 are periodically active when the animal is not feeding (Schactner and Bräunig, 1995) but the SN2 are completely silent. Both neuron pairs are primarily active when the animal is feeding, but the SN1 are active at a slightly higher frequency (Schactner and Bräunig, 1995). Further studies are needed to elucidate the exact roles of serotonin and dopamine. The exact cellular location of the aminergic receptors must be ascertained in conjunction with identifying all of the receptor types for serotonin and dopamine and their transduction mechanisms.

It is likely that there is more than one type of receptor for each amine associated with the salivary glands. If we are to take an example from cockroach and blowfly salivary glands, where dopamine and serotonin bind to 2 classes of receptors respectively, then it is possible that they do the same in the salivary glands of *Locusta migratoria*. We have not tested for their effects on intracellular Ca\(^{2+}\) levels or for their effects on the inositol trisphosphate second messenger system. We also have not tested their ability to directly gate ion channels. In the cockroach, dopamine binds to one receptor which is coupled to adenylate cyclase, and to another which mediates the release of intracellular Ca\(^{2+}\).

Similarly, in the blowfly, serotonin induces a rise in cyclic AMP levels, but also binds to separate receptors which mediate the liberation of IP\(_3\) and intracellular Ca\(^{2+}\) (Prince and Berridge, 1972). It would not be surprising to find that serotonin and dopamine have similar actions in locusts.
Figure 1: Schematic representative model of the stimulus-secretion coupling mechanism in the salivary glands of *Locusta migratoria*. Dopamine induces an elevation of cyclic AMP and possibly (?) calcium in one cell type which leads to the movement of ions and water into the lumen of the acini. Serotonin induces an elevation of cyclic AMP and possibly (?) calcium in a different cell type which leads to the secretion of enzymes and proteinaceous saliva. Middle cell shows the ion (Na⁺, K⁺, Cl⁻) flow via various routes during steady state salivation. Question marks (?) denote unknown events. DA; dopamine; 5-HT: serotonin; cAMP: cyclic AMP. Adapted from Petersen (1992).
Nerve Terminals

Dopamine

Serotonin

Acini Cells

H₂O

K⁺

Ca²⁺ (?)

cAMP

? Cl⁻

H₂O

6 Na⁺

6 Cl⁻

6 Na⁺

6 Cl⁻

Lumen of Acini

Enzyme Release

5-HT
It would be pertinent to give an overall perspective of the complete innervation of the salivary glands of Locusta, including the putative neuroactive substances that might affect salivation. To begin with, the glands are innervated by salivary neurons from the suboesophageal ganglion (SN1 and SN2) that contain dopamine and serotonin as neurotransmitters. The amino acid, GABA, is present in SN2 (Watkins and Burrows, 1989), but nothing is known of the actions of GABA in the salivary glands. The satellite nervous system is found to project to the level of the acini and its activity during feeding suggests that it releases serotonin into the haemolymph surrounding the salivary glands and ducts. The glands are also innervated via branches from the median transverse nervous system which stain positively for FMRFamide-like immunoreactivity. Furthermore, FMRFamide-like peptides are probably associated with the salivary glands (Fusé et al., 1996). The biogenic amine octopamine, has been found associated with the glands and is also found in an unpaired median neuron whose soma lies in the metathoracic ganglion and which sends branches to the salivary glands via metathoracic nerve 1 and then via the transverse nervous system (Bräunig et al., 1995). The peptide, LOM-SG-SASP, has been isolated from the glands and appears to have sialigogic effects, possibly through a cyclic AMP system (Veelaert et al., 1995).

Clearly, the innervation, control and modulation of the salivary glands of the locust is not a simple matter. Are we to believe that we have identified all of the neuroactive substances involved in salivation? I hold a view contrary to this belief. We are only in the initial stages of unravelling the elements that control salivation, and that govern amine-amine and amine-peptide interactions in this preparation. The accessibility of the salivary glands and the neurons which innervate them ensures that this invertebrate preparation will offer many unique insights into various aspects of neurophysiology.
References


