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A LOCUST OCTOPAMINE-IMMUNOREACTIVE DORSAL UNPAIRED MEDIAN NEURONE FORMING TERMINAL NETWORKS ON SYMPATHETIC NERVES

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Summary

In insects, octopamine is present in neurohaemal regions of the thoracic sympathetic nervous system, but its cellular source is unknown. We describe a dorsal unpaired median neurone (DUM1b) in the locust metathoracic ganglion that forms a meshwork of varicose, presumably neurohaemal, endings on the surfaces of sympathetic nerves. Other targets include several ventral longitudinal muscles, the spiracle closer muscle, tissue remnants of degenerated nymphal muscles and the salivary glands. Using an established antiserum, DUM1b is shown to be octopamine-immunoreactive, and its target muscles to be covered with octopamine-immunoreactive varicosities. Octopamine influences one of these muscles in essentially the same way that another well-described octopaminergic neurone, DUMEti, modulates the extensor tibiae muscle of the hind leg. We propose that DUM1b is an octopaminergic modulator of muscle contractions and may also influence numerous other body functions by releasing octopamine as a hormone from sympathetic neurohaemal areas.

Introduction

In insects, octopamine functions as a neurotransmitter, neuromodulator and neurohormone (for reviews, see Evans, 1985; Agricola *et al.* 1988; Orchard *et al.* 1993).

Evidence for a hormonal function has accumulated from numerous studies. Octopamine levels in the haemolymph are raised during locomotor activity (Goosey and

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Candy, 1980) and under stressful conditions (Davenport and Evans, 1984). It can then act as a circulating 'sympathetic' factor increasing, for example, the availability of carbohydrate and lipid substrates (see Evans, 1985). The octopamine present in the haemolymph is assumed to be derived from endocrine organs of the retrocerebral complex as well as from neurohaemal structures associated with the segmentally arranged, unpaired median and transverse sympathetic nerves (Evans, 1985; Myers and Evans, 1988). In locusts, regions of the thoracic median nerve contain octopamine (Evans, 1985), and specific octopaminergic receptors are present on the spiracle muscles, which are innervated by the transverse nerve (Swales *et al.* 1992). In crickets, these nerves are covered with octopamine-immunoreactive processes (Spörhase-Eichmann *et al.* 1992). The cellular origin of octopamine in the thoracic sympathetic nervous system is, however, unknown.

The only identified efferent octopaminergic neurones in insects are members of a population of dorsal unpaired median (DUM) neurones (Evans, 1985). Individually identified DUM neurones have been shown to use octopamine either as a transmitter, for example controlling bioluminescence of the firefly light organ (Christensen and Carlson, 1981), or as a modulator influencing the contractile properties of specific skeletal (Evans and O'Shea, 1977; Whim and Evans, 1988) and visceral muscles (Orchard and Lange, 1985). All efferent DUM cells so far identified in locust thoracic ganglia have axons that project bilaterally into the paired nerves (Watson, 1984; Thompson and Siegler, 1991) and none is known to be associated with the sympathetic nervous system. In this study, however, we describe an octopamine-immunoreactive metathoracic DUM neurone that innervates several skeletal muscles through axons in paired nerve 1 and axon collaterals in mesothoracic sympathetic nerves. These nerves and target muscles are covered with a dense meshwork of varicose endings from this DUM cell. Our findings suggest that this DUM neurone modulates muscular contractions and may, in addition, influence numerous other body functions by releasing octopamine as a hormone into the circulation from neurohaemal areas. Some of the results have been published in abstract form (Stevenson and Bräunig, 1993).

Materials and methods

Experimental animals

All experimental animals, adult migratory locusts (*Locusta migratoria* L.), were taken from established crowded colonies kept under standard conditions.

Intracellular recording and cobalt staining

After anaesthetizing locusts by chilling, the head, legs and abdomen were removed and the thorax bisected above the spiracles into dorsal and ventral halves. The ventral side was pinned dorsal side up and the thoracic ganglia and all relevant peripheral nerves exposed. A small Sylgard resin platform was then placed under the metathoracic ganglion for support, and the preparation was superfused with locust Ringer (as described by Clements and May, 1974, but without sucrose). Neurones were impaled in their somata with glass microelectrodes filled with cobalt hexammine chloride ($0.1\,mol\,l^{-1}$; d.c.

resistance 40–60 MΩ). Impaled neurones were physiologically identified by a 1:1 correlation of soma action potentials with extracellular action potentials recorded from peripheral nerves with hook electrodes. Recordings were amplified by standard techniques.

Cobalt ions were introduced into impaled cells by applying depolarizing current pulses (5–15 nA, 200 ms at 2.5 Hz for 30–60 min). After cobalt injection, peripheral nerves on one side of the ganglion were squashed to allow more cobalt to diffuse into the contralateral nerves during subsequent incubation (12 h, 4 °C). Cobalt was precipitated with ammonium sulphide (0.3 %) and preparations were washed in Ringer before being placed in alcoholic Bouin's fixative (2 h). Staining was then intensified with silver (Bacon and Altman, 1977). For viewing, ganglia were dehydrated and cleared in methyl salicylate.

Immunocytochemistry

Octopamine-immunoreactive processes were revealed in paraffin sections (10 μm) and whole-mount preparations using an established antiserum. Dot blot immunoassay has shown this antiserum to be highly specific for octopamine, but slightly cross-reactive with tyramine; tissue staining is blocked by preincubating the antiserum with appropriate octopamine conjugates, but not with tyramine conjugates (Eckert *et al.* 1992). Bound antiserum was detected by either the standard peroxidase/anti-peroxidase (PAP; Sternberger, 1979) or avidin biotin methods, using diaminobenzidine (DAB) as chromogen according to protocols given elsewhere (Stevenson *et al.* 1992, 1994). No staining is observed when the primary antiserum is omitted.

An identified DUM neurone was shown to be octopamine-immunoreactive by a double-staining procedure. A severed nerve branch containing the DUM cell's axon was placed in a Vaseline pool filled with distilled water. This was refilled after 5 min with the tracer Neurobiotin (Vector Laboratories, 2 % in Ringer) and the preparation was left in a humidity chamber (18–48 h, 20 °C). Ganglia were then processed for immunocytochemistry. Alternate 5 μm paraffin sections of the same ganglion were mounted on separate glass slides. One set was processed by the PAP technique to detect octopamine, and the other with peroxidase-coupled avidin biotin complex (Vector Laboratories, 1:100, 2 h) to detect Neurobiotin-filled cells.

Muscle mechanograms and octopamine application

Muscle M87 (terminology of Snodgrass, 1929) was dissected free, together with attached cuticle, and transferred to a 0.5 ml bath continuously superfused with aerated isotonic Ringer (Clements and May, 1974). The cuticle was pinned down and the free apodeme glued to a thread attached to a force transducer (Cambridge Instruments) to measure tension almost isometrically. The nerve supply was stimulated using a suction electrode, with the intensity adjusted so as to just evoke contractions. The Ringer could be exchanged for Ringer containing DL-octopamine (Sigma) using a manually operated two-way tap.

Terminology

Nerves are numbered, and some muscles named according to Campbell (1961); other

muscle are numbered according to the system of Snodgrass (1929, see the legend to Fig. 2 for abbreviations).

Results

An identified DUM neurone projecting to sympathetic nerves

The sympathetic nervous system in thoracic segments is derived from the unpaired median nerve, which arises from the posterior dorsal midline of each thoracic ganglion and divides almost immediately into the left and right transverse nerves (Fig. 1A). Our observations of the finer branching pattern of these nerves (Fig. 2) conformed to descriptions given elsewhere (Campbell, 1961; Myers and Evans, 1988; Baines *et al.* 1989). The aim of this paper is to identify octopaminergic dorsal unpaired median (DUM) neurones projecting into this system of peripheral nerves.

In the metathoracic ganglion, we identified a dorsal midline neurone that produced long-lasting overshooting soma action potentials (amplitude 50–60 mV), typical of efferent DUM cells, in response to electrical stimulation of either the left or right mesothoracic transverse nerve (Fig. 1B, upper trace). These stimuli also evoked an extracellular potential at a constant latency in the transverse nerve contralateral to that stimulated (Fig. 1B, lower trace). In these experiments, the transverse nerve was cut proximal to the site of stimulation so that the closer motoneurones of the mesothoracic spiracles, which bifurcate in the left and right transverse nerves (Miller, 1960), were not activated.

A corresponding dorsal midline neurone was physiologically identified in nine separate animals. Subsequent cobalt staining and silver intensification always revealed a neurone with the morphological characteristics of a DUM cell: a large dorsal soma (diameter 40–50 μm) and a bifurcating primary neurite giving rise to bilaterally projecting axons in left and right nerve 1 (N1; Figs 1C, 2). In all nine cases, we observed the same basic pattern of peripheral axon branching. After entering N1, the axon sends collaterals to the recurrent nerve and nerve branch N1A, before continuing in the main root of N1 (Fig. 1C). It should be noted that the point where N1A branches off from N1 often varies, even in the same animal (Fig. 1C, compare left and right N1A). Further distally, the axon then turns to enter N1B and all of its branches (Fig. 2). As already shown by the electrophysiological recordings, this DUM neurone also sends axon collaterals into the transverse nerve through an anastomosis with N1B (Figs 2, 4A). One collateral runs proximally towards, but not reaching, the median nerve and enters side branches of the transverse nerve that appear to connect with the suboesophageal salivary gland nerve. A second axon collateral in the transverse nerve projects distally, to the spiracle muscles, and sends a branch to N1B2a (Fig. 2).

The DUM neurone that we recorded and stained was never found to have an axon in N1D, through which DUMDL (Hoyle, 1978), otherwise named DUM1a (Goodman and Bate, 1981), projects to the dorsal longitudinal flight muscle. Furthermore, and in accordance with findings in crickets (Davis and Alanis, 1979), DUMDL (identified by recording its extracellular action potential in N1D) was not found to produce action potentials in the recurrent nerve, N1A or N1B. The neurone we describe must therefore

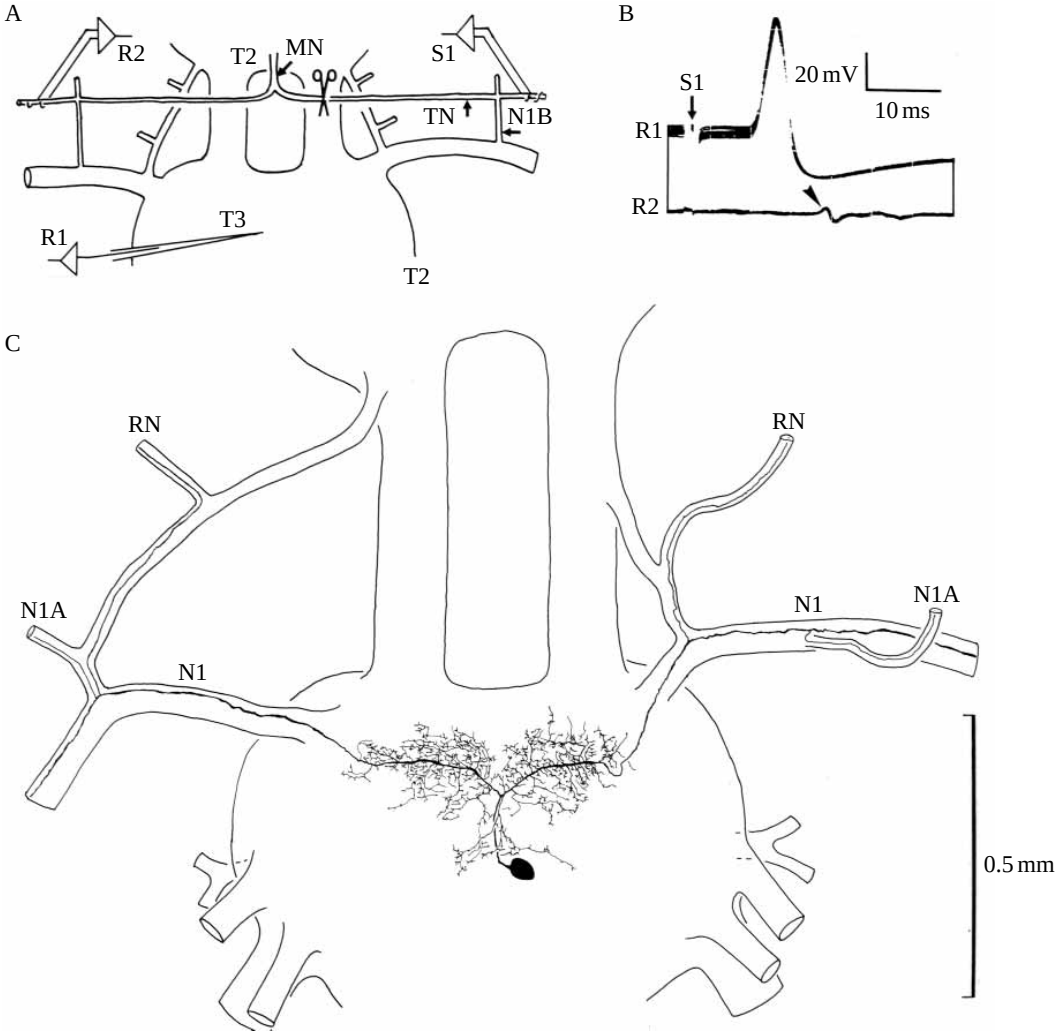

Fig. 1. Identification of DUM1b. (A) Sketch of experimental design. DUM cells were recorded intracellularly (R1) in the metathoracic ganglion (T3) and extracellular hook electrodes were placed on the left and right branches of the mesothoracic transverse nerve (TN), which are derived from the median nerve (MN), for either extracellular recording (R2) or antidromic stimulation (S1). Note, TN proximal to S1 was severed to prevent antidromic activation of efferent neurones in T2. (B) Multiple sweeps showing action potentials of DUM1b recorded from the soma (R1, top trace) and left branch of TN (R2, lower trace, arrowhead) evoked by stimulating right TN (S1, arrow). (C) *Camera lucida* drawing of a silver-intensified cobalt fill of DUM1b. Note the bilaterally symmetrical axon projections into the recurrent nerve (RN) and N1A, features that distinguish this cell from DUM1a (DUMDL). The axon remaining in N1 continues to the more distally located N1B, through which it enters the TN (see Figs 2 and 4A). Scale bar, 0.5 mm.

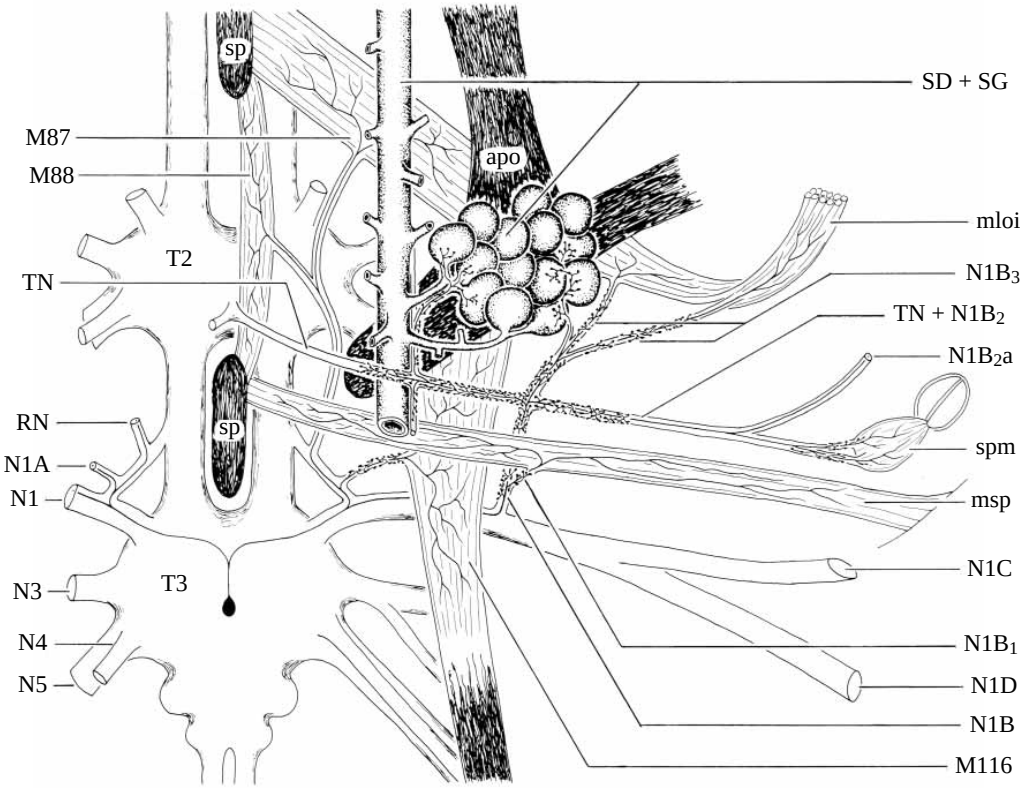

Fig. 2. Semi-schematic drawing of principal peripheral projections and targets of DUM1b, as revealed by silver-intensified cobalt staining after removing most of the salivary gland tissue. apo, apodeme; M, muscle; mloi, mesothoracic lateral oblique intersegmental muscle; msp, mesothoracic spina-pleural muscle; N, nerve; RN, recurrent nerve; SD, salivary duct; SG, salivary gland; sp, spinum; spm, mesothoracic spiracle muscle; T2, T3, mesothoracic and metathoracic ganglia respectively; TN, transverse nerve.

be DUM1b, which has been described in the locust embryo as the sibling of DUMDL and is the only other DUM cell with axons in N1 (Goodman and Bate, 1981).

Electrophysiological experiments also indicated that a corresponding DUM cell exists in the mesothoracic ganglion. This neurone has similar peripheral connections through N1 to the prothoracic transverse nerve (data not shown). In the prothoracic ganglion, however, a neurone corresponding to DUM1b was not found. In this ganglion, only one octopamine-immunoreactive axon (Stevenson *et al.* 1992) and only one DUM cell project into nerve 1. This neurone appears to be the prothoracic homologue of DUM1a (P. Bräunig, unpublished results).

Peripheral targets of DUM1b

Cobalt staining also revealed fine varicose endings (diameter 0.7–2.6 μm) of DUM1b on several skeletal muscles (Figs 2 and 3). These include (1) the ventral longitudinal

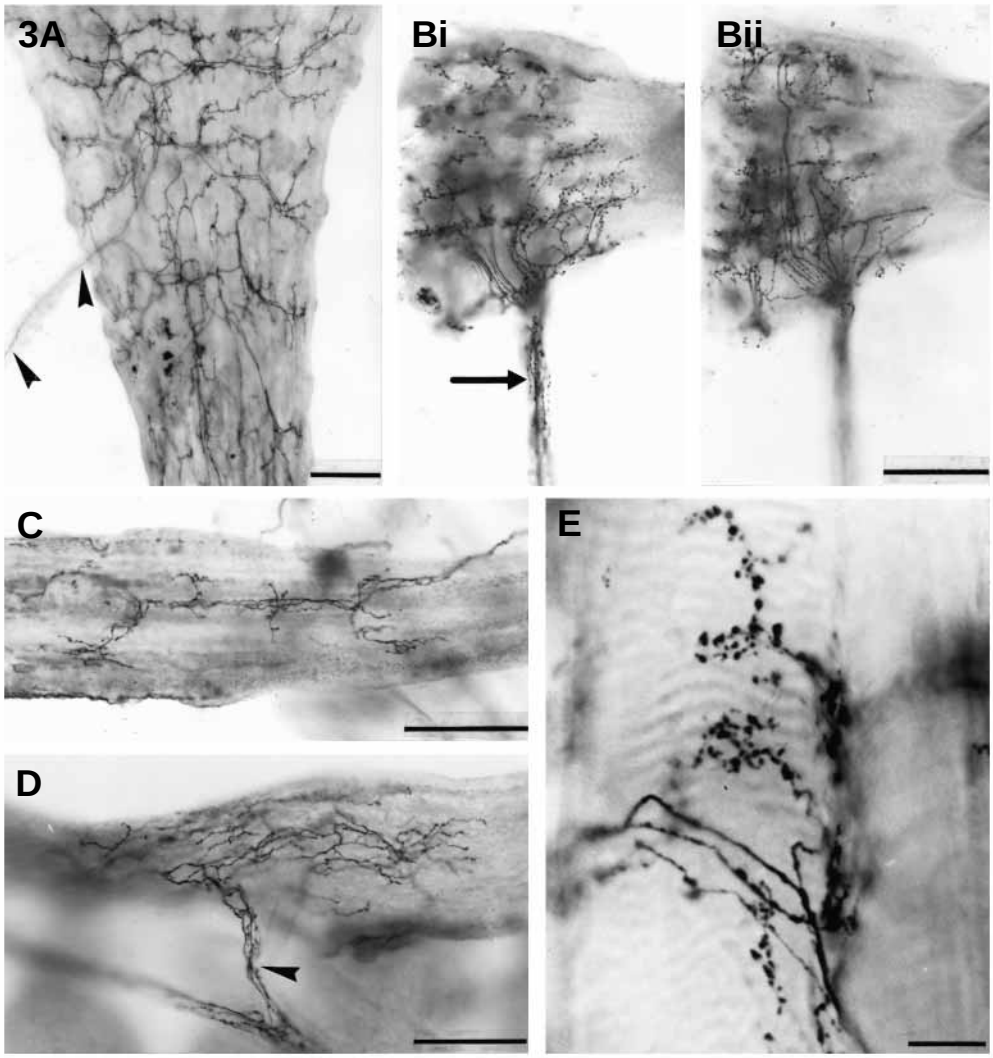

Fig. 3. Photomicrographs of terminal axon projections of DUM1b onto target muscles, revealed by intracellular cobalt staining. (A) M116, arrowheads indicate axon in N1A. (Bi,ii) Mesothoracic spiracle muscle in two focus planes. Note varicose endings surrounding the transverse nerve (Bi, arrow). (C) Mesothoracic spina-pleural muscle. (D) Mesothoracic lateral oblique intersegmental muscle of young adult locust (compare with Fig. 4C). Arrowhead indicates varicosities on $N1B_3$. (E) Detail of M87 (compare with Fig. 5C). Scale bars, A, B, C, D, 100 μm; E, 20 μm.

muscles M87 (Fig. 3E) and M88, innervated by the recurrent nerve, and M116 (Fig. 3A), which is innervated by N1A; (2) the nymphal mesothoracic spina-pleural (Fig. 3C) and lateral oblique intersegmental muscles (Fig. 3D), which are still present in young adult locusts, where they are innervated by branches of N1B; and (3) the closer muscle of the mesothoracic spiracle (Fig. 3B), which is innervated by the transverse nerve. DUM1b

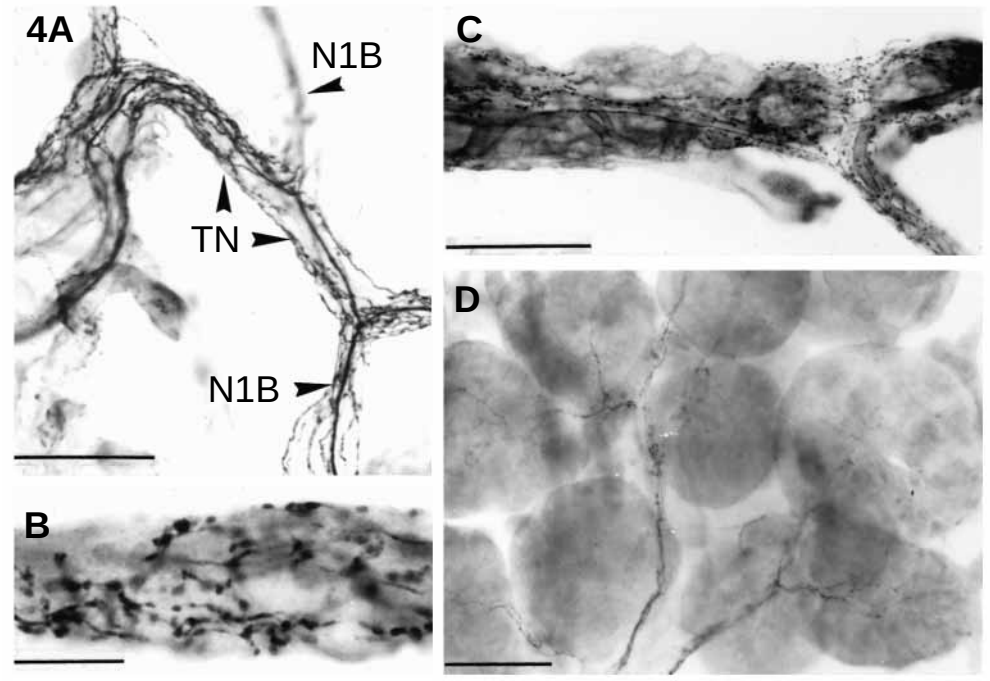

Fig. 4. Photomicrographs of putative neurohaemal release sites of DUM1b, revealed by intracellular cobalt staining. (A) A DUM1b axon passing through the right N1B and into the transverse nerve (TN) through an anastomosis. Note the varicose processes surrounding these nerves. (B) Detail of varicose endings around TN. (C) DUM1b endings on the surface of N1B3 and on the connective tissue remnant of the degenerated lateral oblique intersegmental muscle of an old mature adult locust. (D) DUM1b projections to the acini of the salivary glands. Scale bars, A, C, D, 100 μm; B, 20 μm.

may also project through N1B2a to the mesothoracic pleural subalar muscle (Campbell, 1961), but we could not see this in our preparation.

In contrast to other identified DUM cells, DUM1b establishes a dense meshwork of varicose endings surrounding the sheaths of several nerves. Such processes envelop large portions of the transverse nerve and N1B (Figs 2, 4A,B), as well as distal regions of N1A, N1B1, N1B3 and the transverse nerve just before they reach their respective target muscles (Figs 2 and 3). Furthermore, although the mesothoracic spina-pleural and lateral oblique intersegmental muscles degenerate in mature adult locusts (Campbell, 1961), their connective tissue remnants are covered with varicose endings of DUM1b (Fig. 4C). Similar processes occur in the salivary glands, where they branch over individual acini (Fig. 4D). These arise from DUM1b projections in side branches of N1B3 and the transverse nerve (Fig. 2).

Immunocytochemistry of DUM1b and its targets

Retrograde Neurobiotin staining of the branch of the recurrent nerve that innervates M87 labelled a single, dorsal midline soma in the metathoracic ganglion (Fig. 5A). This

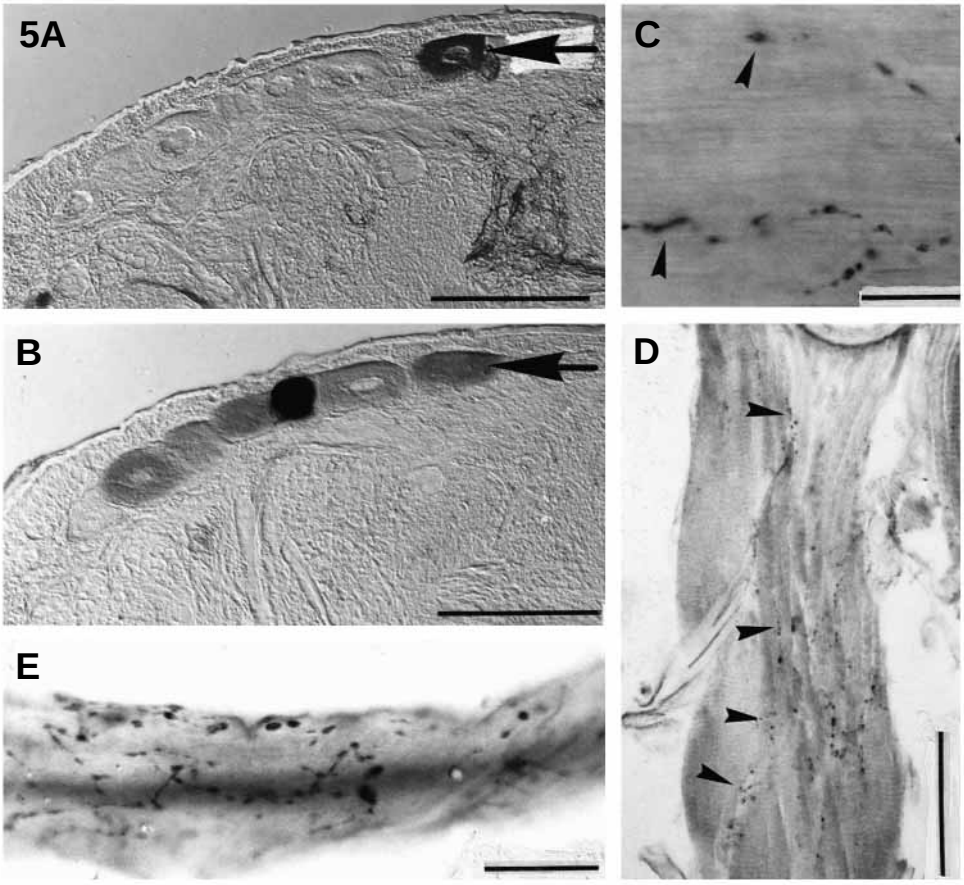

Fig. 5. Photomicrographs of octopamine-immunoreactive labelling of DUM1b and its targets. (A) Midline sagittal section through the dorsal anterior region of the metathoracic ganglion showing DUM1b soma (arrow) stained by backfilling the recurrent nerve with Neurobiotin (anterior to the left, dorsal to the top). (B) Section adjacent to that shown in A, processed for octopamine immunocytochemistry, showing that DUM1b is octopamine-immunoreactive (arrow). (C) Octopamine-immunoreactive processes (arrowheads) on M87. (D) Spiracle muscle. Arrowheads mark octopamine-immunoreactive varicosities. (E) The transverse nerve. Scale bars, A, B, D, 100 μm; C, E, 20 μm.

neurone can therefore correspond only to DUM1b. Adjacent ganglion sections, processed either to reveal Neurobiotin-filled somata (Fig. 5A) or to reveal octopamine immunoreactivity (Fig. 5B), clearly showed DUM1b to be octopamine-immunoreactive.

Each of DUM1b's target muscles was found to be covered with octopamine-immunoreactive varicosities, which form chains running between, and also crossing, muscle fibre bundles (Fig. 5C,D). In regions of peripheral nerves and salivary tissue innervated by DUM1b, background staining was typically very high and in most cases we were unable to discern any clear octopamine-immunoreactive label. Nevertheless, we occasionally observed octopamine-immunoreactive varicosities surrounding the transverse nerve in favourable whole-mount preparations (Fig. 5E), but unfortunately

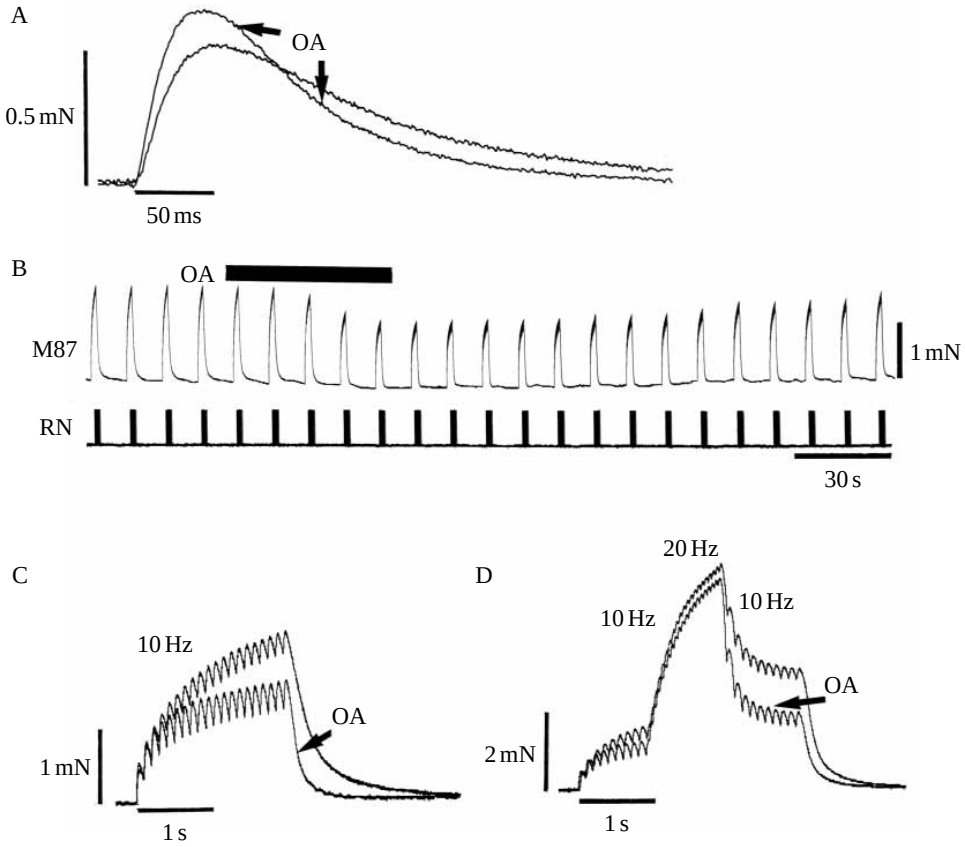

Fig. 6. Modulation of contractile properties of a DUM1b target muscle. (A) Two superimposed single twitch contractions of M87, one prior to, and one during, bath superfusion with 10^{-6} mol l^{-1} DL-octopamine (OA, arrows); note the increases in twitch amplitude, contraction velocity and relaxation velocity caused by octopamine. (B) Relaxing effect of octopamine (OA, 10^{-6} mol l^{-1}, bar marks application) on tension evoked by brief 10 Hz trains of stimulation of the recurrent nerve (RN). (C) Two superimposed sweeps taken from B, one prior to the addition of octopamine, the other during octopamine superfusion (OA, arrow). (D) Two superimposed sweeps showing catch-like tension in M87 and its reduction by octopamine (OA, arrow). The muscle was stimulated at 10 Hz with an interposing sequence at 20 Hz.

these were not seen in sections. The density of octopamine-immunoreactive varicosities around the transverse nerve (30 000–50 000 mm^{-2}) and on the spiracle muscle (15 000–20 000 mm^{-2}) was in the same range as that found for cobalt stainings of DUM1b. Their size, however, was slightly smaller (diameter 0.3–2.0 μm), which is probably due to greater shrinkage of tissue during preparation for immunocytochemistry. Furthermore, we observed no labelling in axons or between varicosities, so that the general appearance of immunocytochemical staining was less dense than that expected from cobalt labelling.

Response of a DUM1b target muscle to octopamine

The effects of octopamine on evoked contractions of a target muscle of DUM1b (M87, Fig. 6) matched in all major respects those documented for the extensor tibiae muscle (Evans and O'Shea, 1977; Evans and Siegler, 1982). For example, bath application of 10^{-6} mol l^{-1} DL-octopamine increased the amplitude of single twitch contractions of M87 by approximately 23 % (Fig. 6A). This was accompanied by an increase in the speed of contraction and relaxation (Fig. 6A). Resting tension and tetanic tension, however, were both reduced by octopamine (Fig. 6B,C). Octopamine also reduces catch-like tension in M87, which develops following an interposing period of higher-frequency stimulation (Fig. 6D).

Discussion

The locust metathoracic ganglion probably contains 20 octopaminergic efferent DUM cells (Siegler *et al.* 1991; Stevenson *et al.* 1992). Although the basic morphology of several has been described (Watson, 1984), the specific peripheral targets and functions are known for essentially only two: DUMETi (Hoyle *et al.* 1974; Evans and O'Shea, 1977) and DUMDL (Hoyle, 1978; Whim and Evans, 1988), which modulate contractions of the extensor tibiae and the dorsal longitudinal flight muscle, respectively.

This paper describes the peripheral targets of metathoracic DUM1b, identified in locust embryos as the sibling of DUMDL, and the only other DUM cell with axons in N1 (Goodman and Bate, 1981). This cell has previously been observed in adult locusts (Altman and Tyrer, 1977) and crickets (Bartos and Honegger, 1992), but has not otherwise been characterized. A homologous neurone is probably present in the mesothoracic ganglion, and other homologues may be present in abdominal ganglia 1–7, where two octopamine-immunoreactive DUM cells project into N1 (Stevenson *et al.* 1992, 1994; Stevenson and Pflüger, 1994).

In most respects, DUM1b is like other efferent DUM cells. It is octopamine-immunoreactive and projects to several skeletal muscles. All these muscles are covered with octopamine-immunoreactive processes. One (the spiracle closer muscle) possesses specific octopaminergic receptors (Swales *et al.* 1992) and another (M87) is modulated by octopamine in the same manner that DUMETi influences the extensor tibiae muscle (cf. Evans and O'Shea, 1977; Evans and Siegler, 1982). Thus, as shown for DUMETi, we propose that DUM1b is octopaminergic and functions to change the response of muscle to efferent control from a response that favours maintaining tension to one that favours rapid changes in tension.

Apart from the spiracle muscle, which is active during respiration (Miller, 1960), the functions of DUM1b's target muscles are not known. The attachment sites of the ventral longitudinal muscles are suited to moving the prothoracic segment relative to the mesothoracic segment. Other target muscles span non-articulated exoskeleton and may serve only to maintain body shape in newly moulted locusts (Campbell, 1961) or to assist ecdysial movements, ventilatory pumping or haemolymph flow.

Unlike other identified DUM cells, DUM1b establishes a dense meshwork of varicose endings covering tissue remnants of degenerated nymphal muscle, branches of N1 and

the mesothoracic transverse nerve. Similar profiles near the thin neural sheath of the transverse nerve have been revealed by cobalt staining (Myers and Evans, 1988; Baines *et al.* 1989), the glyoxylic acid method for detecting catecholamines (Myers and Evans, 1988), as well as with antibodies against various peptides (Myers and Evans, 1985; Garcia-Scheible and Honegger, 1989) and octopamine (Spörhase-Eichmann *et al.* 1992; this study). In all cases, these structures have been likened to neurohaemal release sites in stick insects (Finlayson and Osborne, 1968). DUM1 may, therefore, influence a wide variety of body functions by releasing octopamine into the general circulation as a hormone, or as a factor controlling the release of other hormones.

Projections of DUM1b through the transverse nerve to the salivary glands suggest a specific function in influencing secretory activity of the acini. In cockroaches, octopamine potentiates neurally evoked secretory potentials (Bowser-Riley and House, 1976). In locusts, studies of the aminergic innervation and modulation of salivary glands have focused on serotonin and dopamine (Baines and Tyrer, 1989; Baines *et al.* 1989; Ali *et al.* 1993), although octopamine has been detected in these organs (Robertson and Juorio, 1976).

The DUM1b neurone is the first identified cellular source of octopamine in thoracic sympathetic nerves. Octopamine antisera have revealed neither central neurones projecting directly into the median nerve nor peripheral cell bodies on sympathetic nerves (Eckert *et al.* 1992; Spörhase-Eichmann *et al.* 1992; Stevenson *et al.* 1992, 1994). We would not, however, entirely dismiss the possibility that such octopaminergic cells exist. Some peripheral neurosecretory cells take up Neutral Red, indicating an amine content (Myers and Evans, 1988), and lateral neurones entering the median nerve (Myers and Evans, 1985) are positioned in a similar way to several octopamine-immunoreactive somata in thoracic ganglia (see Stevenson *et al.* 1992). Nevertheless, since only N1 is connected with the transverse nerve (Campbell, 1961), DUM1b is probably the only thoracic DUM cell innervating this target.

Clearly, however, all efferent DUM cells may release octopamine as a hormone. DUM cells in the suboesophageal ganglion project to the retrocerebral complex and its associated nerves (Bräunig, 1990, 1991), forming terminal ramifications similar to those seen for DUM1b. Others may form peripheral octopaminergic networks surrounding paired nerves, such as are found in crickets (Spörhase-Eichmann *et al.* 1992). Finally, DUM cell targets, such as the heart (Ferber and Pflüger, 1990), antennal heart (Pass *et al.* 1988; Bräunig, 1990), oviducts (Orchard and Lange, 1985; Kalogianni and Pflüger, 1992) and even skeletal muscle, can also be considered to be neurohaemal release sites, rather than just effector organs. Future studies must determine the behavioural conditions during which DUM1b is activated, and whether such activity produces any hormonal effects that accord with the neurosecretory function of this neurone suggested by the findings presented here.

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