

SHORT COMMUNICATION

SULPHIDE SILVER STAINING FOR ENDOGENOUS HEAVY METALS REVEALS SUBSETS OF DORSAL UNPAIRED MEDIAN (DUM) NEURONES IN INSECTS

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The central nervous systems of many insects contain segmentally arranged groups of unpaired midline neurones. These have been called DUM (dorsal unpaired median) neurones because of the position of their cell bodies. The larger DUM neurones, with bilaterally symmetrical peripheral axons, have been most extensively studied in grasshoppers and locusts. DUM cells in the metathoracic ganglion modulate tension of skeletal muscle, potentiate synaptic transmission at neuromuscular junctions and slow a myogenic rhythm (Evans and O'Shea, 1977, 1978; Whim and Evans, 1989), whereas those in the abdominal ganglia alter tension in visceral muscle (Lange and Orchard, 1984). In fireflies, DUM neurones of the terminal abdominal ganglia control flashing by activating the photocytes of the lantern (Christensen and Carlson, 1981). The effects are mediated by the biogenic amine octopamine (Carlson and Jalenak, 1986; Evans and O'Shea, 1977, 1978; Morton and Evans, 1984; Orchard and Lange, 1985), which is also implicated in other modulatory actions (see Ramirez and Orchard, 1990, for references). In grasshoppers, in addition to the octopamine-containing efferent DUM neurones, there are a substantial number of local and intersegmental DUM interneurones with small cell bodies (Thompson and Siegler, 1989, 1991).

The most widely used method for revealing putative octopamine-containing neurones in insects has been to stain with the vital dye Neutral Red (e.g. locust, Evans and O'Shea, 1978; cockroach, Dymond and Evans, 1979; firefly, Christensen and Carlson, 1981). This dye is not specific for octopamine, but stains biogenic amine-containing cells (Stuart *et al.* 1974); if these do not fluoresce in tests for catecholamines, the inference is that they may contain octopamine (see Evans, 1985, for a discussion of the technique). We have had equivocal results using Neutral Red to stain DUM neurones in grasshopper ganglia, however, with widespread nonspecific staining of neurones, poor contrast and rapid loss of the stain. An immunocytochemical study has reported the use of an antiserum raised against octopamine to stain locust DUM neurones (Konigs *et al.* 1988). This antibody does not, however, stain other cells reported to be octopaminergic in the

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locust (Orchard and Loughton, 1981) and the reproducibility of the staining of DUM neurones with this antibody is questionable (P. D. Evans, personal communication). Here we report that the Timm sulphide silver reaction permanently and selectively stains the large DUM neurones in insect ganglia. This histochemical reaction is probably revealing endogenous copper, present as a cofactor to tyramine beta-hydroxylase, the enzyme that synthesizes octopamine.

Experiments were performed on last instar and adult grasshoppers *Schistocerca americana* (Dirsh) and last instar cockroaches *Periplaneta americana* L. from laboratory colonies, and on adult fireflies *Photuris* sp. captured from the wild in Atlanta in June and July of 1990. Ganglia were removed from the insects and treated for 10 min with a few drops of ammonium sulphide in 5 ml of insect saline to precipitate endogenous heavy metals. Ganglia were fixed in 10 % formalin for 1 h, taken from 70 % alcohol to distilled H₂O in a graded series, then developed immediately or soaked in Timm's developer base for up to 24 h at room temperature or 48 h at 4°C. The developer base contained 6 g of gum acacia, 20 g of sucrose, 0.86 g of citric acid and 0.34 g of hydroquinone in 200 ml of distilled water (pH 3.0), with or without 0.5 % Triton X-100 (modified from Bacon and Altman, 1977; see Siegler and Pousman, 1990). After warming the ganglia to 50°C, silver nitrate was then added to a concentration of 0.05 % (w/v), and the ganglia were incubated in the dark at 50°C until DUM neurone cell bodies were darkly stained against a paler background, usually after 45 min to 2 h. Ganglia were transferred to fresh solution every 30 min.

DUM cell bodies were dark within a pale ganglion when the developer base did not contain Triton X-100 and when developed immediately without presoaking; darkening occurred throughout the neuropile when Triton X-100 was present, lessening the contrast for the cell bodies as viewed in whole mounts, but providing more complete stains of axons and neuropilar processes. Ganglia were dehydrated, cleared and examined in whole-mount or Epon sections (8 µm). Some ganglia were pretreated for 1 h in 15 % trichloroacetic acid (TCA), then rinsed thoroughly before development, to dissolve FeS but leave CuS intact, as described by Holm (1989). The method was developed for grasshoppers, where DUM neurones stained selectively in over 80 % of the preparations (*N*=90 animals). In the remaining preparations, either all neuronal cell bodies darkened together, or the neuropile darkened so rapidly that it was impossible to distinguish the staining of the overlying DUM neurone cell bodies.

In the mesothoracic ganglion of the grasshopper, the stained cell bodies of the DUM neurones lie at the posterior end of the ganglion (Fig. 1A). The primary neurites of the DUM neurones extend forward, then bifurcate symmetrically, ultimately giving rise to axons in peripheral nerves. Sections reveal staining in the cytoplasm of the DUM neurone cell bodies (Fig. 1B). The tight clustering of the cell bodies makes precise counts difficult, but there are at least 18 and no more than 21 stained DUM cell bodies in the mesothoracic group. Darkening elsewhere in the ganglion occurs in the perineural glia and the cortical glia, which invest the neuronal cell bodies, and in the neuropile (Fig. 1A). The latter results primarily

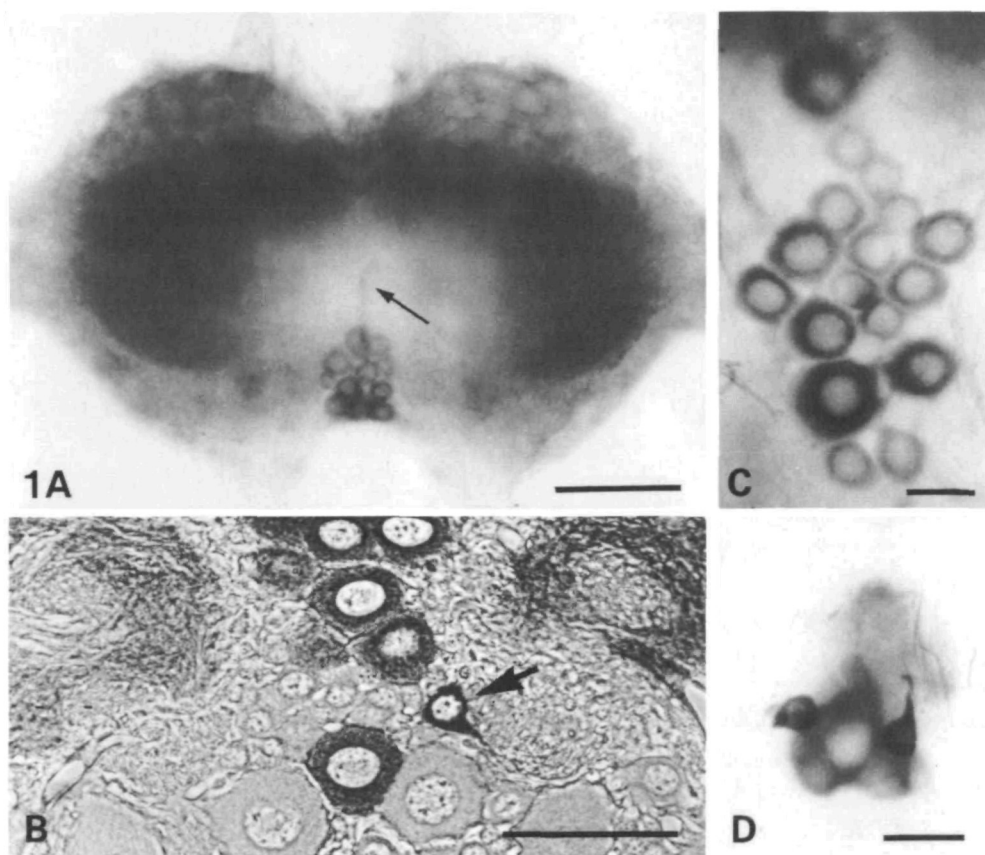


Fig. 1. Photomicrographs of sulphide-silver-stained grasshopper neurones. (A) Dorsal view of a mesothoracic ganglion whole mount, showing the cluster of stained DUM neurones, the bundle formed by their primary neurites and the bifurcation into axons (arrow) at the midline. (B) Transverse section through the DUM group in the mesothoracic ganglion showing the cytoplasmic location of stain in cell bodies, staining in the lateral regions of the neuropile, and the contrast between stained large DUM cell bodies and unstained cell bodies of small DUM neurones and motor neurones. One of the stained midline pair of neurones also appears in the section (arrow). (C) Close-up of stained DUM neurones viewed dorsally in a whole mount of the metathoracic ganglion. (D) Close-up of the stained midline pair viewed ventrally in a whole mount of the mesothoracic ganglion. Scale bars: 200 μm (A); 100 μm (B); 50 μm (C,D).

from staining of the processes of the DUM neurones, and is most pronounced in the lateral regions of the neuropile (Fig. 1A,B). In the metathoracic ganglion, the stained cell bodies occur within four midline groups of DUM neurones, one associated with each of the neuromeres. The largest group, in the third thoracic (T3) neuromere, lies at the midline near the anterior and contains 20 or 21 stained cell bodies (Figs 1C, 2A,B). Each abdominal group (Ab1–3) contains three stained cell bodies (Fig. 2A,B). In every case, the stained cell bodies were the

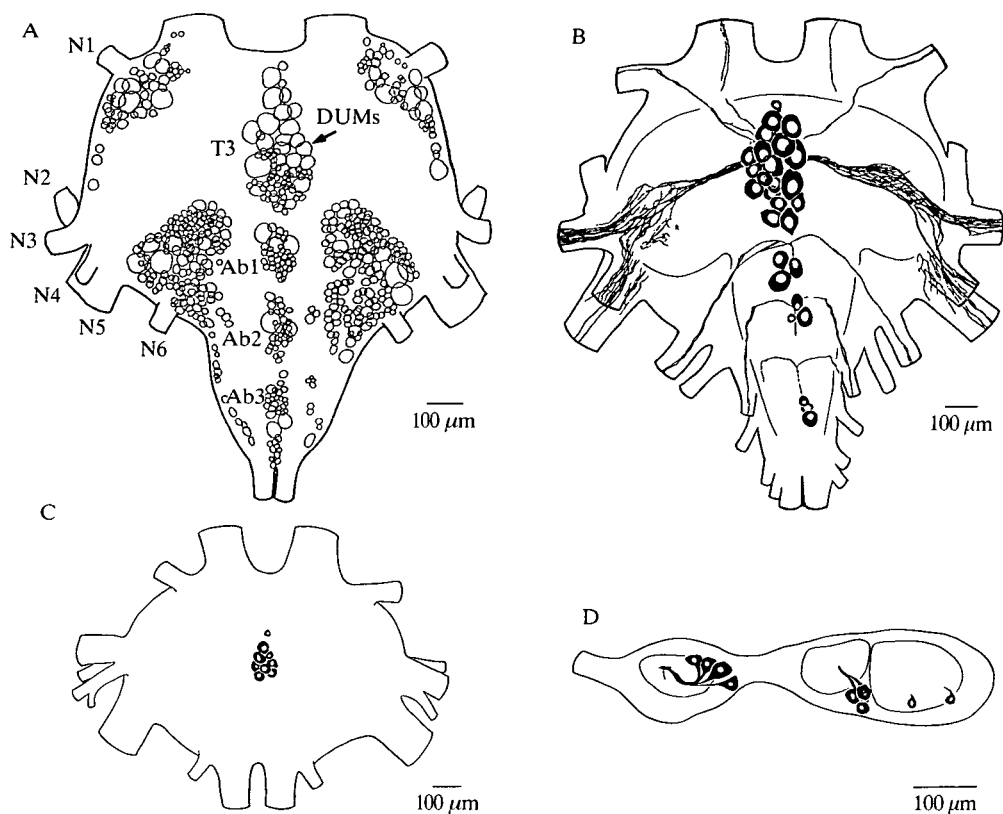


Fig. 2. DUM neurones in a grasshopper, a cockroach and a firefly. (A) Neuronal cell bodies in the dorsal cortex of the grasshopper metathoracic ganglion, drawn from a Toluidine-Blue-stained whole mount. The four groups of DUM neurone cell bodies are: T3, third thoracic, and Ab1–3, first to third abdominal neuromeres. B–D are *camera lucida* drawings of sulphide-silver-stained neurones in whole mounts of (B) grasshopper metathoracic ganglion; (C) cockroach metathoracic ganglion; and (D) terminal abdominal ganglia A6 (left) and A7 (right) of a firefly.

largest in the group. The primary neurites of the DUM neurones enter the neuropile beneath the cell bodies and bifurcate to travel laterally (Fig. 2B). At least two axons exit in N1 and N6 and four or more axons exit in N3, N4 and N5 on each side of the ganglion. The primary neurites and axons are beaded and of fine calibre, however, making it difficult to trace all of them for any individual neurone. A small number of other unidentified cell bodies were also stained. The most darkly stained of these was a small midline pair, ventral to the DUM neurones and outside the DUM group. A similar pair was stained in the mesothoracic ganglion, and in the T3, Ab1 and Ab2 neuromeres of the metathoracic ganglion (Fig. 1B,D). Faintly stained cell bodies occurred more laterally, but were too variable in number (1–3 in any location) to identify routinely. Neuronal cell bodies elsewhere in the ganglia, including the many small

neurones that comprise the remainder of the DUM groups, did not darken more than the background (Fig. 2A,B). The large DUM neurones are efferents, whereas the small DUM neurones, studied in T3, are local or intersegmental interneurons (Thompson and Siegler, 1989, 1991). This selectivity of staining cannot be explained by differences in cell body size (and thus cytoplasmic volumes), inasmuch as cell bodies of the midline pair, outside the DUM group, are similar in diameter (approx. 20 μm) to those of the small DUM neurones, but stain darkly (Fig. 1B). Excitatory motor neurones and the common inhibitors, which have cell bodies comparable in size to those of the large DUM neurones, are also unstained.

In the cockroach metathoracic ganglion, staining revealed eight DUM cell bodies at the midline of the T3 neuromere, along with one more-anterior unidentified neurone (Fig. 2C), and a midline pair (not shown). Eight large efferent DUM neurones have been reported in this ganglion (Tanaka and Washio, 1988). In the firefly, unpaired midline neurones were stained in abdominal ganglia 6 and 7 (Fig. 2D); these were similar in size and position to the octopamine-containing neurones that innervate the lantern (Christensen and Carlson, 1981; Christensen *et al.* 1983).

Sulphide silver staining appears to present a useful alternative to Neutral Red staining for revealing putative octopamine-containing neurones in insects. The present method is a modification of histochemical techniques used to detect heavy metals in mammalian brain and spinal cord by precipitation of metal sulphides and subsequent physical development (see Holm, 1989). Two heavy metals, iron and copper, are cofactors of enzymes in the biosynthetic pathways of the biogenic amines and are possible sources of the reactivity described here. Iron is a cofactor in the conversions of tryptophan to 5-hydroxytryptophan (by tryptophan 5-hydroxylase), of phenylalanine to tyrosine (by phenylalanine hydroxylase) and of tyrosine to DOPA (by tyrosine hydroxylase). However, the pattern of staining of the midline cells was the same in ganglia treated with TCA, which removes FeS reactivity, and in untreated ganglia. Copper, therefore, seems the more probable source of the heavy metal reactivity. It is a cofactor of both tyramine beta-hydroxylase (TBH), the synthetic enzyme for octopamine (Wallace, 1976), and of dopamine beta-hydroxylase, the synthetic enzyme for noradrenaline. The staining would, therefore, be correlated with the presence of octopamine or noradrenaline in the midline neurones. Octopamine seems to be the more likely candidate, as DUM cells have been shown to contain octopamine in the locust, cockroach and firefly (Evans and O'Shea, 1978; Dymond and Evans, 1979; Christensen *et al.* 1983). No adrenergic neurones have been identified in insects, the ganglia contain 10 times less noradrenaline than octopamine and no detectable adrenaline (Evans, 1980), and no physiological role for noradrenaline or adrenaline is known.

The selective staining of the large but not the small DUM neurones in the grasshopper draws attention to a problem of developmental interest, that of diversity within a neuronal lineage group. All the DUM neurones in a segment, 80–90 neurones in T3 for example, are the progeny of a single unpaired median

neuroblast (Goodman *et al.* 1980). Only 20 of these show sulphide silver staining, and perhaps these alone contain octopamine and function as efferent modulatory neurones. (Ten individual large DUM neurones have been identified; Watson, 1984.) The many remaining small neurones in the T3 lineage group are local or intersegmental interneurones (Thompson and Siegler, 1989, 1991). These small DUM neurones contain GABA-like immunoreactivity whereas the large DUM neurones do not (Thompson and Siegler, 1989). Together these observations support the hypothesis that the DUM lineage group contains distinct subpopulations: efferent modulatory neurones and interneurones that are possibly inhibitory in function.

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