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Multinucleate Neurons with Neurohaemal and Synapsing Axons at the Heart and Alary Muscles of the Butterfly *Caligo beltrao* Illiger (Lepidoptera)

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Summary. The segmental heart nerves of *Caligo beltrao* Illiger (Brassolidae) were examined by transmission and scanning electron microscopy. Heart and alary muscles are innervated by branching processes of single multinucleate neurons (MNNs). There is one MNN situated at each segmental fan-shaped group of alary muscles. The main nerve of the MNN consists of a bundle of processes. This nerve extends centripetally toward the CNS and corresponds to the dorsal portion of the transverse nerve. However, neither axo-somatic nor axo-axonic synapses were found, the presence of which might suggest that this nerve contains axons of different neuronal origin. The synaptic contacts of the MNN with axons originating from the CNS are therefore assumed to be established beyond the spiracular region.

In addition to the neuro-muscular junctions of the smaller centrifugal axon branches there are neurohaemal release sites along the entire length of all MNN axon bundles. Axon terminals are packed with either dense-cored or multigranular vesicles. Both morphological types of vesicles are, however, found side by side in the large axons and in the perikaryon, often at the same Golgi element. These morphological findings may support the concept that more than one transmitter is produced in a single neuron. Questions that arise in reference to dual or polyfunctional neurons and to the control of cardiac activity are discussed.

Key words: Transmitter-dualism – Multipotential neuron – Heart innervation – Ultrastructure – SEM.

An electron microscopic study of the innervation of the heart and alary muscles of adult *Sphinx ligustri* (Sphingidae) and *Ephestia* (Pyralidae) suggested that all

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synapsing and neurohaemal nerve terminals originate from the transverse segmental heart nerves (Wasserthal and Wasserthal 1977). In this report, the swellings of the heart nerves were thought to contain cell bodies that are homologous to the peripheral neurosecretory cells in the transverse nerve of caterpillars observed by Hinks (1975) under the light microscope. For the elucidation of the neural control of cardiac activity the following questions regarding heart nerve morphology had to be answered: Do the neurohaemal and neuromuscular axon terminals originate from different nerve cell bodies or from one and the same cell? What relationship exists between the presumed peripheral neurosecretory cells and the transverse nerve? Since the cardiac nerves are easier to detect in living preparations of the relatively large owl butterfly *Caligo beltrao* than in the moths mentioned above, the present study was carried out in this species.

Materials and Methods

The insects were obtained as chrysalids from a breeder in Brazil and emerged in an air conditioned room (23°C, 90% rh), where they were allowed to fly freely and were fed ripe bananas. They reached an age of 3 months. Most of the tissue examined was taken from two active males about 2 months old. After fixation of the exposed abdominal content, the part of the nerve supply of the heart extending from the spiracle region to the corresponding cardiac segment including the nerve swelling, the adhering alary muscle fibres and the adjacent part of the heart itself were excised. The preparations were serially sectioned either perpendicular or parallel to the heart "nerve" and the cell body. Fixation and embedding for TEM and REM were the same as described earlier (Wasserthal and Wasserthal 1977). The specimens were examined in a Zeiss EM 9S and an ISI S III A respectively.

Results

Cell Body of the Multinucleate Neuron

Each transverse segmental heart nerve shows a swelling near the site of attachment of the nerve to the alary muscles, which in the 6th and 7th abdominal segments lie much closer to the heart than in the more anterior segments (Fig. 1). This swelling proved to contain the perikaryon of a single multinucleate neuron (MNN). The rounded nuclei are evenly distributed within the cytoplasm (Fig. 3). The existence of a large lobate nucleus of the kind frequently found in polyploid insect cells can be ruled out because there are no connections between the nuclei. The presence of about 16 to 32 rounded nuclei could be confirmed by phase contrast microscopy in whole mount preparations.

The perikaryon of the MNN possesses loosely arranged rough endoplasmic reticulum (rER) (Fig. 4). At the periphery of the Golgi bodies two types of vesicles can be observed: dense-cored vesicles of about 120–180 nm \varnothing and multigranular vesicles of 160–250 nm with heterogeneous electron dense content (Figs. 4, 5). The perikaryon is incompletely enveloped by glial cells. The cell body shows pseudopodiallike outgrowths interdigitating with the glial envelope (Fig. 7). The cytoplasm of the outgrowths sometimes contains rER. Parts of the MNN cell surface are directly adjacent to the haemocoel, and are covered only by extracellular stromal material (Fig. 6). The glial cells send complex processes into the MNN cell

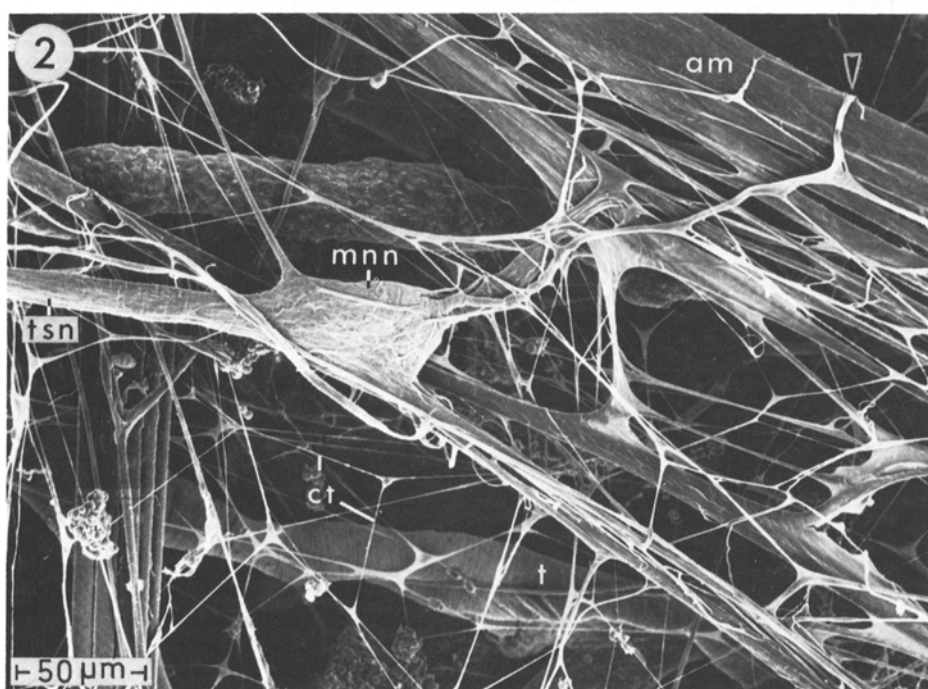
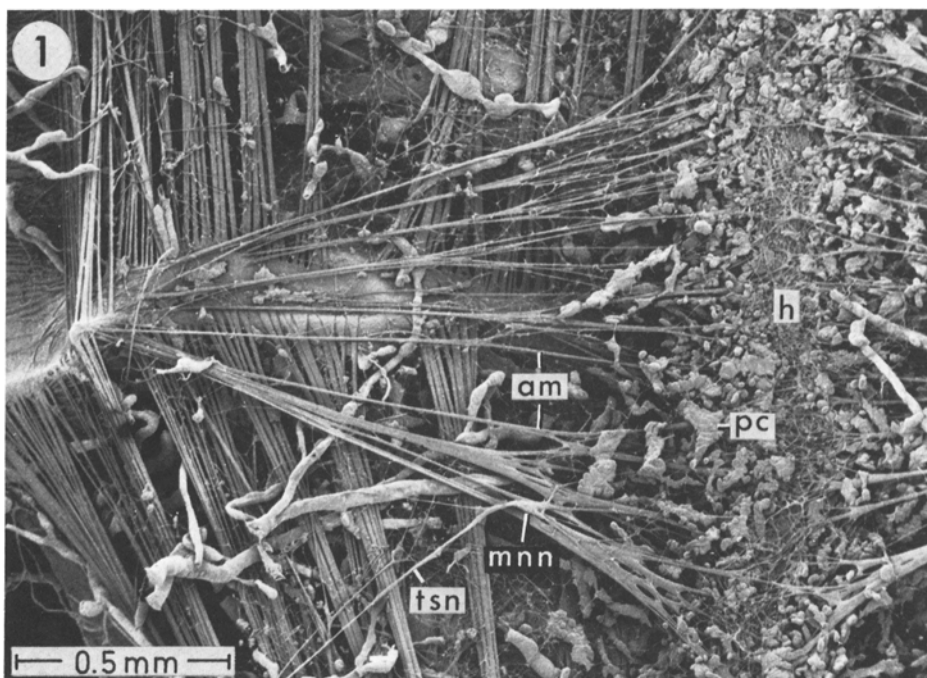


Fig. 1. Multinucleate neuron (*mnn*) of 6th abdominal segment of *Caligo beltrao* ♂, supplying alary muscles (*am*) and heart (*h*). Centripetal process of neuron (i.e., dorsal portion of transverse segmental heart nerve, *tsn*) extends toward spiracular region. Heart tube covered by network of branching alary muscles and pericardial cells (*pc*). SEM. $\times 56$

Fig. 2. Multinucleate neuron (*mnn*). Note small centrifugal axon bundle (arrowhead) attached to alary muscle (*am*); *ct* connective tissue, *t* trachea, *tsn* transverse nerve; SEM. $\times 340$

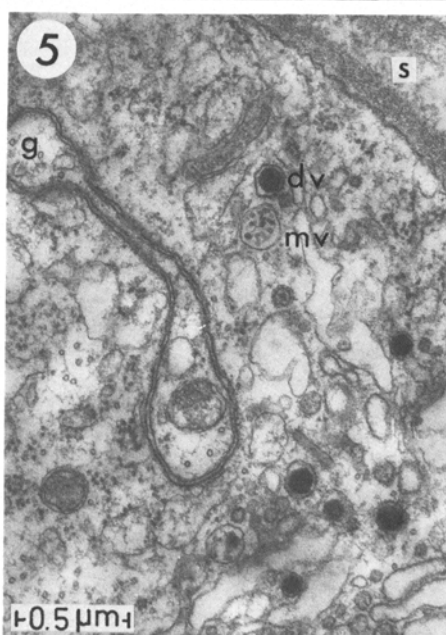
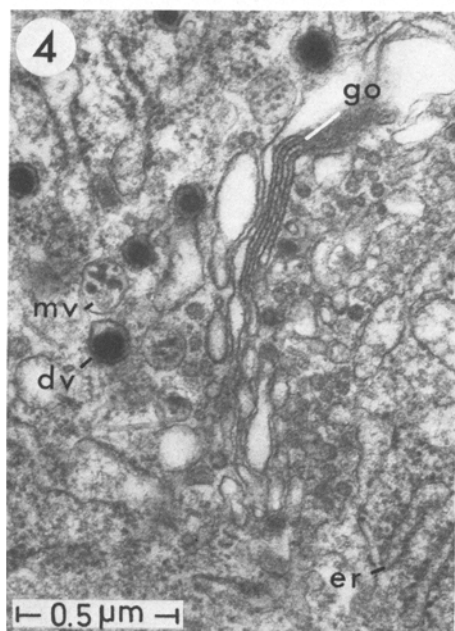
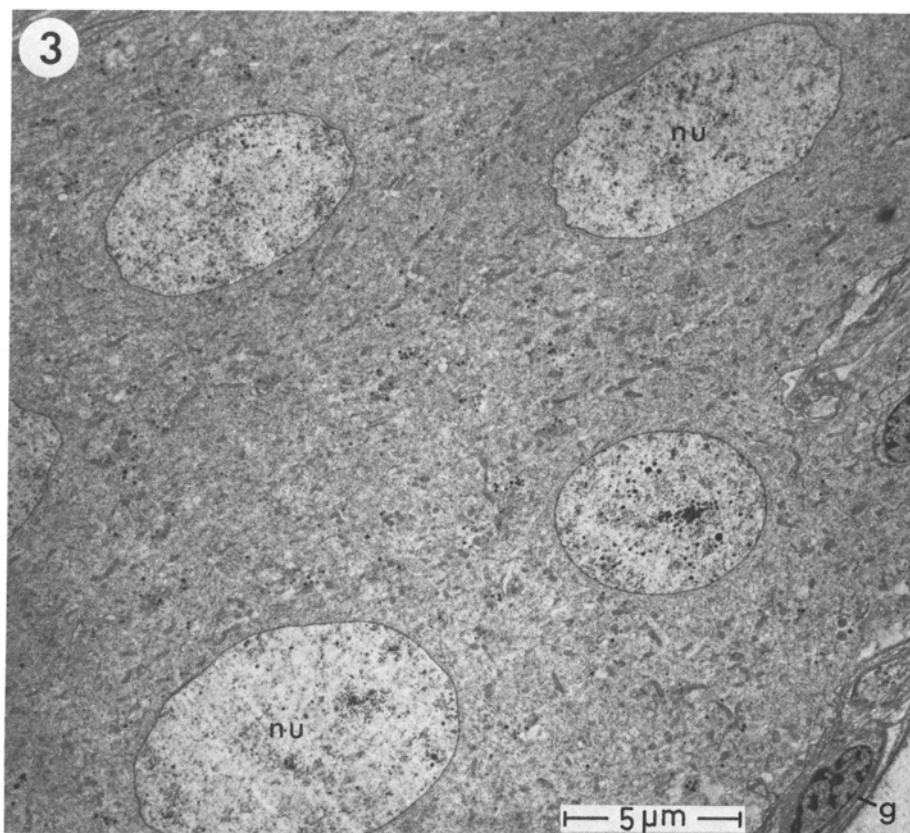


Fig. 3. Perikaryon of multinucleate neuron with scattered isolated nuclei (*nu*); *g* glial cell. $\times 4600$

Fig. 4. Cytoplasm of MNN with dense-cored vesicles (*dv*) and multigranular vesicles (*mv*) near Golgi complex (*go*); *er* endoplasmic reticulum. $\times 42,000$

Fig. 5. Surface of MNN covered only by extracellular stromal material (*s*); *dv* dense-cored vesicle, *g* glial invagination (trophosphonium), *mv* multigranular vesicle. $\times 30,000$

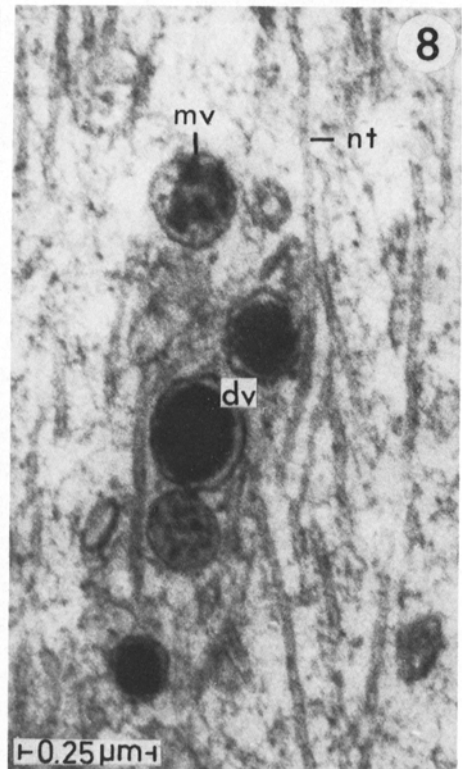
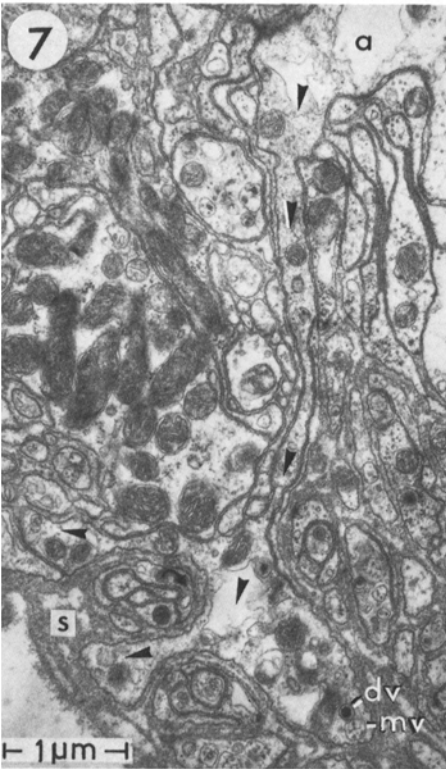
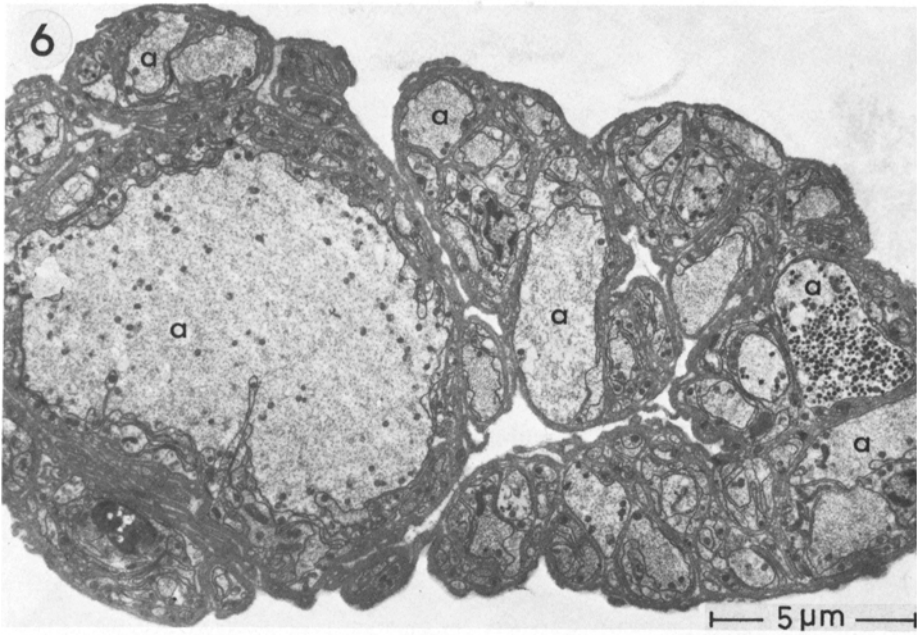


Fig. 6. Transverse seminal nerve in cross section halfway between MNN cell body and spiracular region; note main process and smaller branches (*a*) originating from MNN. $\times 5360$

Fig. 7. Outgrowths of main process of MNN (*a*) penetrating glial sheath and reaching outer surface of transverse segmental nerve (arrowheads); *dv* dense-cored vesicle, *mv* multigranular vesicle, *s* extracellular stroma. $\times 16,000$

Fig. 8. Main process with dense-cored (*dv*) and multigranular vesicles (*mv*); longitudinal section; *nt* neurotubules. $\times 72,000$

body (trophospongium). The arrangement and ultrastructure of the glial cells will be described in more detail separately.

Processes of the MNN

The cytoplasm of the perikaryon of the MNN continues into that of the transverse nerve. This connection was traced in serial longitudinal and in cross sections of the transverse segmental nerve near the swelling: The transitional zone between the MNN cell body and the main (centripetal as well as centrifugal) processes still contains ribosomes and rER and also abundant neurotubules. Whereas the diameter of the central process within the centripetal MNN nerve (the transverse nerve) remains rather constant along its entire length, the arrangement, diameter, and number of peripheral axons vary within short distances due to repeated branching (Fig. 6). Axon bifurcations were frequently seen in longitudinal sections. No axo-axonic or axo-somatic synapses were found. In the larger processes, dense-cored as well as multigranular vesicles are loosely scattered (Fig. 8). The peripheral axon branches and the neurosecretory terminals are densely packed with either dense-cored or multigranular vesicles (Figs. 9–11). Except for single vesicles of one type within groups of vesicles of the other type, mixed populations of both types were not observed in the terminals.

Neurohaemal Release Sites

The axons and glial sheaths of the transverse nerve do not form a tight bundle but are loosely arranged, leaving spaces filled with extracellular stroma or even areas of free haemocoel (Figs. 6, 9). This arrangement of glial cells is also found at the axon terminals filled with neurosecretory vesicles and here it is a prerequisite for the extrusion of the neurosecretory material. Accumulations of small vesicles (20 nm or less) below the plasma membrane covered only by extracellular stroma are frequent among the dense-cored vesicles of the axon terminals (Fig. 10). This configuration is characteristic of neurosecretory release sites (Smith 1970; Normann 1974; Scharrer and Wurzelmann 1978). Clear vesicles occur sporadically in the axon terminals containing the multigranular vesicles. The neurosecretory axon terminals are evenly distributed along the length of the main bundle of MNN processes. Small neurohaemal release sites are also found along the synapsing axon branches, sometimes directly beside neuro-muscular junctions. These contain only dense-cored vesicles.

Synaptic Innervation of Alary Muscles and Heart

The branches of the centrifugal processes of the MNN are attached to the alary muscles by connective tissue (Fig. 2). They often accompany the alary muscles within a common sheath of connective tissue as they extend toward the heart. The neuro-muscular junctions at the alary muscles, however, were found only in the area of pericardial cells, that is, in proximity to the heart and at the alary-muscle sheath around the heart. While the neuro-muscular junctions are abundant at the

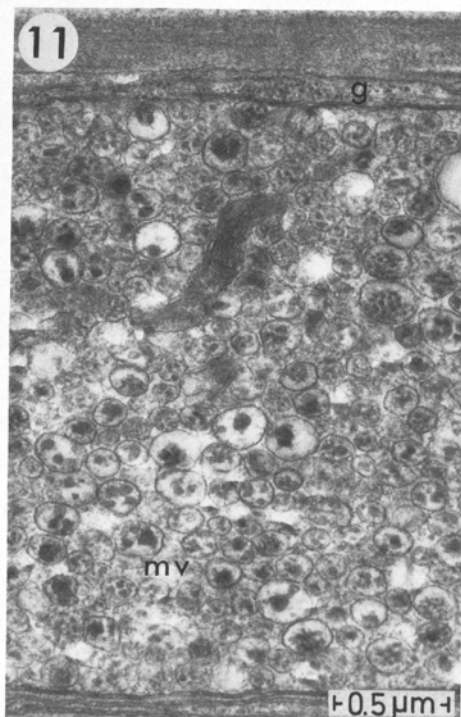
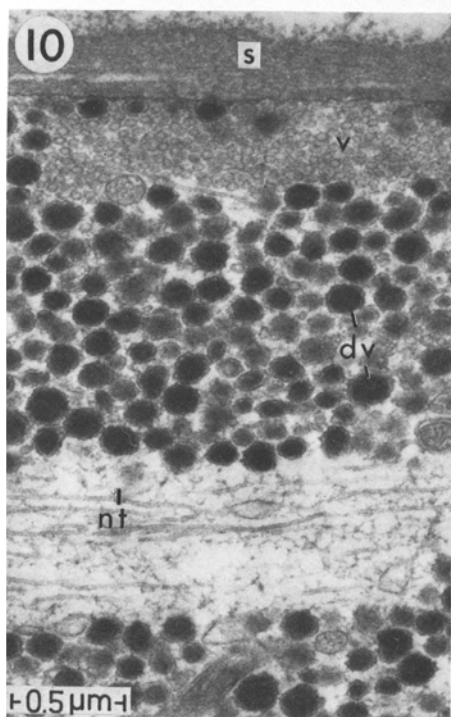


Fig. 9. Separate accumulation of dense-cored vesicles (*dv*) and multigranular vesicles (*mv*) in different axon terminals (*at*) of transverse segmental nerve in cross section; *a* axon, *g* glial cell, *s* stromal layer. $\times 18,500$

Fig. 10. Neurosecretory axon terminal with dense-cored vesicles (*dv*); presumed release site with clear vesicles (*v*); longitudinal section; *nt* neurotubules, *s* stromal layer. $\times 30,000$

Fig. 11. Axon terminal with multigranular vesicles (*mv*) showing variable content; *g* glial process. $\times 30,000$

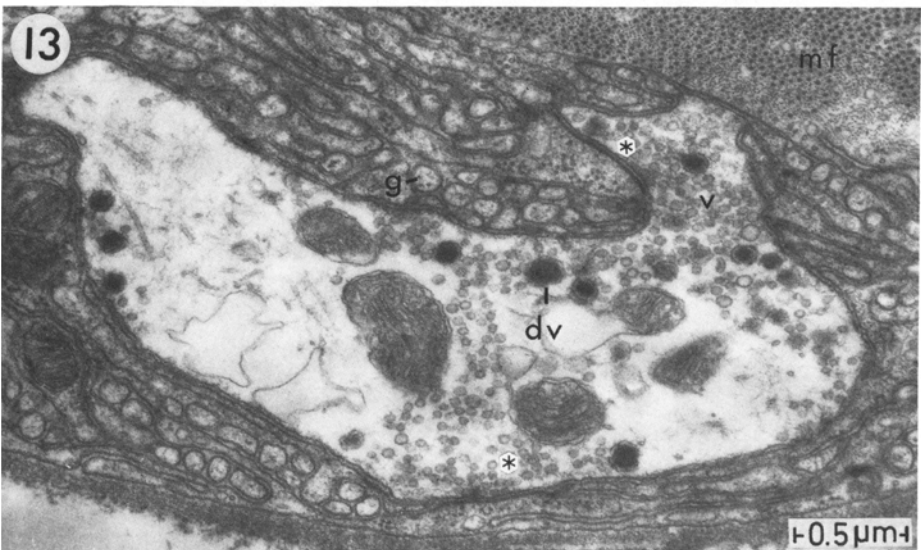
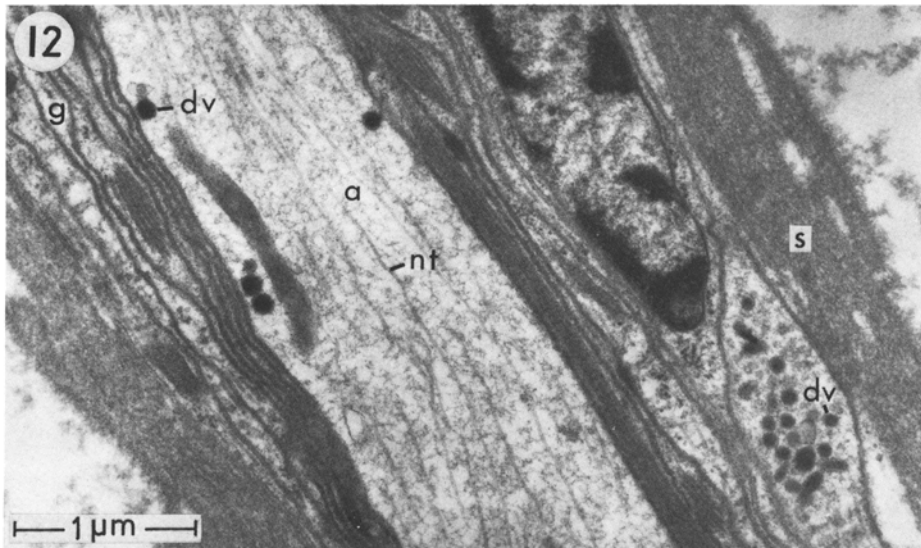


Fig. 12. Axon near heart with dense-cored vesicles (*dv*) and presumed neurohaemal release site in longitudinal section; *g* glial envelope, *nt* neurotubules, *s* stromal layer. $\times 24,000$

Fig. 13. Synapsing (*) axon terminal at alary muscle fibre with interdigitations of glial cell (*g*); *dv* dense-cored vesicle, *mf* myofibrils, *v* synaptic vesicles. $\times 30,000$

alary muscles and at the posterior tip of the heart, they are rare at the other heart muscle cells. The neuro-muscular contacts are characterized by typical synaptic vesicles near presynaptic densities (Fig. 13). They also contain dense-cored vesicles of the same size as described above. The organization of the neuro-muscular junctions is almost the same as that in the *Sphinx* moth heart. The same kind of

interdigitations occurs between glial cells and muscle cells (Wasserthal and Wasserthal 1977; Rheuben and Reese 1978). The glial insulation, which is rather imperfect along most of the length of the MNN processes, becomes tight and multilayered around the axons in the area of neuro-muscular junctions (Fig. 12). As in the *Sphinx* moth, an inner glial cell type with dense bundles of microtubules (gII, according to Maddrell and Treherne 1967) is also found here.

Discussion

In *Periplaneta* the heart is innervated by separate cardiac neurosecretory and motor neurons as well as directly and indirectly by neurosecretory axons descending segmentally from the ganglia of the ventral nerve cord (Miller 1973). In the butterfly *Caligo*, however, single peripheral multinucleate neurons (MNNs) give rise to all motor and neurosecretory axon terminals at the alary muscles and at the heart. Each MNN also gives rise to the bundle of processes that corresponds to the dorsal portion of the transverse segmental nerve (TSN). All MNN processes contain neurosecretory material from the perikaryon. The MNN is probably homologous to the "group of neurosecretory cells" at the TSN in moth caterpillars described by Hinks (1975). He proposed that these cells are connected with neurosecretory axons of different, more centrally located neurons. However, neither axo-axonic nor axosomatic synapses could be found in either the dorsal portion of the TSN or at the MNN which might confirm the presence of axons of different neuronal origin in that part of the TSN. We therefore assume that the connection between MNN and CNS (or peripheral interneurons) is established laterally at the spiracular region or even ventral to it. The presence of two morphologically different types of neurosecretory vesicles and of both neurohaemal and neuromotor terminals of the MNN means that the structural and functional complexity of the nerve supply to the lepidopteran heart refers to single neurons. This is remarkably different from the intricate arrangement of various types of nerve cells found in cockroaches.

The distribution of the dense-cored and multigranular vesicles – side by side near the Golgi elements in the MNN perikaryon but separated in the neurohaemal axon terminals (Fig. 14) – may suggest that after formation the two morphologically distinct types of vesicles become segregated before entering the different axon terminals. The different appearance of the vesicles, combined with their selective accumulation at the neurohaemal release sites, speaks for the presence of two types of neurosecretory material. This would contradict the generally accepted concept that each nerve cell synthesizes only one transmitter which it uses at all of its terminals (Dale 1933). Other support for the two-transmitter-hypothesis comes from recent embryological investigations. A two-transmitter-stage (acetylcholine and catecholamines) has been found in morphologically undifferentiated neuronal cell cultures, both transmitters being stored in dense-cored vesicles (Schubert and Klier 1977). This establishes, in principle, that two transmitters can be produced by one neuron. Physiological evidence that such conditions may persist in the adult has been reviewed by Burnstock (1978). It is as yet not understood, however, how such a neuron in the differentiated state should work effectively. In molluscs with giant neurons duality of chemical messengers has been explained by their resulting from the fusion of several neuroblasts (Brownstein et al. 1974). In Lepidoptera the

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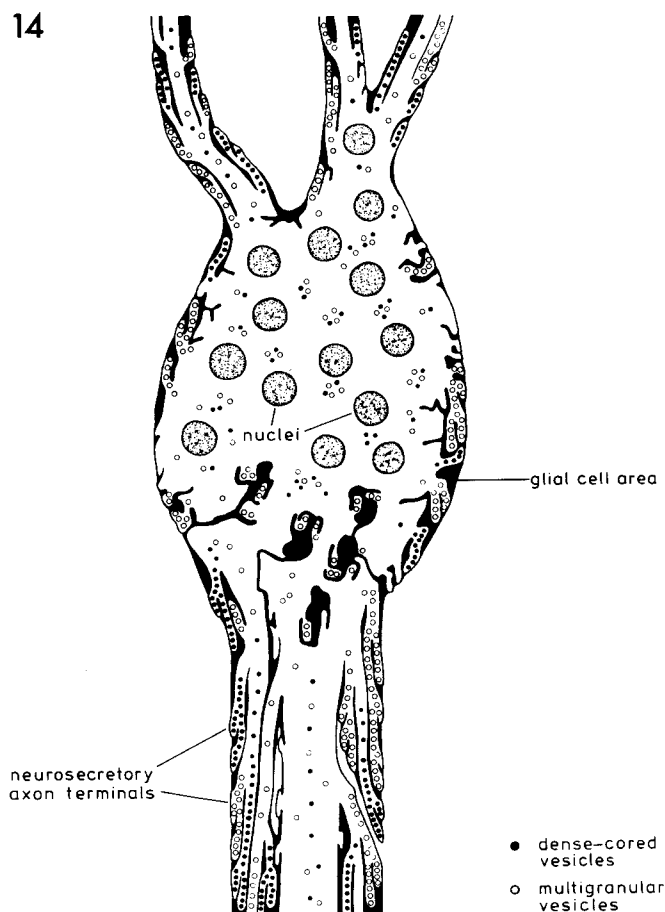


Fig. 14. Diagram of peripheral multinucleate neuron with its one centripetal and several centrifugal bundles of branching processes and axon terminals with neurohaemal release sites

multinucleate state of the MNN may result from nuclear division without cell division.

Even if the two morphologically different types of vesicles should prove to contain the same neurosecretory product, the mode of action of the MNN remains complex because of the combination of neurohaemal release sites and neuromuscular contacts (Fig. 15). The same chemical mediator may, however, signal transmitter-like or neurohormonally. Similar bifunctional neurons seem to be in operation in insects. Axons with neuromotor and neurosecretory terminals have been found at the ventral abdominal intersegmental muscles of *Rhodnius prolixus* (Anwyl and Finlayson 1973). A combination of neurohaemal and integrative function via direct synaptic contacts has been discussed in reference to the ingluvial ganglion of *Blabera craniifer* (Chanussot 1972) and the innervation of the midgut of *Schistocerca gregaria* (Anderson and Cochrane 1977, 1978).

The possibility of a dual or polyfunctional MNN raises the question if and how it receives different signals for different activities. Since all processes of the MNN

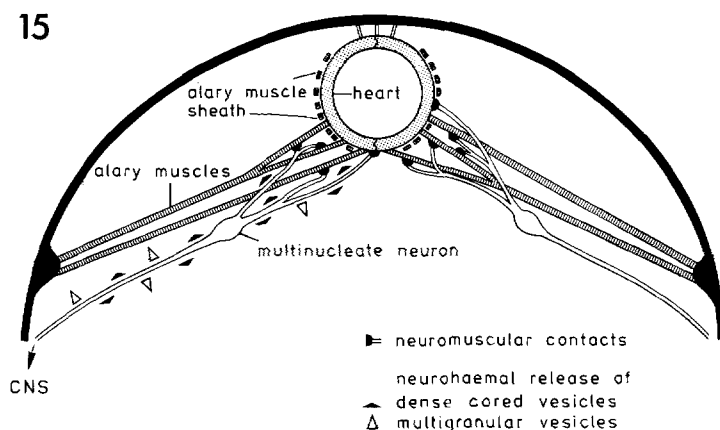


Fig. 15. Diagram of suggested relationships between peripheral multinucleate neuron, heart, and alary muscles

including the TSN seem to have efferent functions, the MNN is possibly an autonomous neuron. Although one should expect central nervous control over the MNN, it is not clear in which way information from the CNS is received. The electrophysiological properties of the MNN may be affected by stretching during periodic changes in abdominal length and/or by changes in the ionic or humoral milieu of the haemolymph during one ventilatory sequence. The MNN seems uniquely exposed to periodic changes in tension brought about by changes in abdominal length because it is strung between the spiracular region and the attachment sites at the alary muscles. Comparable relationships have been reported recently. Stretch receptor neurons have been described in the abdominal segmental nerves of *Carausius morosus* (Orchard and Finlayson 1976). Stretch proprioceptors in the locust thorax show axonal splitting into a meshwork of arborisations with an incomplete glial sheath reminiscent of that in the MNN (Bräunig and Hustert 1980). The possibility of stretch-dependent neurosecretory activity has been considered in a multinucleate neuron having direct neurosecretory contacts with the hindgut muscles of larval *Manduca sexta* (Reinecke et al. 1978). In *Caligo* the differing organization of the glial sheath along the main processes of the MNN (incomplete cell layers) and along the branches near the attachment sites on the muscles (complete layers of two cell types) may indicate specific mechanical but also insulatory properties. The MNN cell body with its incomplete glial sheath may be directly affected by changes in haemolymph CO_2 concentration during each ventilatory sequence. The ability of neurons to respond to increased concentrations of CO_2 in the haemolymph is an important characteristic which serves to regulate abdominal ventilatory movements via the first abdominal ganglion in the cockroach *Byrsotria* (Myers and Retzlaff 1963).

Neural control of the lepidopteran heart has not yet been demonstrated electrophysiologically (McCann 1965). Apart from the morphological results regarding heart and alary muscle innervation there is physiological evidence for neural influence on cardiac activity. The heart of resting *Caligo* periodically changes beating direction (Wasserthal 1975), and these heartbeat reversals are

coordinated with active changes in abdominal length similar to those observed in *Attacus atlas* and in *Papilio machaon* (Wasserthal 1976, and in press). We propose that at rest the MNNs coordinate cardiac activity with the changes in abdominal length and especially serve in the control of the heartbeat during activity.

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