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SHORT COMMUNICATION

ALLATOTROPIN IS A CARDIOACCELERATORY PEPTIDE IN *MANDUCA SEXTA*

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Allatotropin is a neuropeptide that was originally isolated from the sphinx moth *Manduca sexta,* in which it stimulates the synthesis of juvenile hormone in adults (Kataoka *et al.* 1989). An antiserum raised against this peptide was characterized, shown to be specific for allatotropin and used in a competitive enzyme-linked immunosorbent assay (ELISA) to demonstrate that allatotropin is present not only in the brain and retrocerebral complex but also in the ventral nerve cord of *Manduca sexta* (Veenstra and Hagedorn, 1993). In the present study, allatotropin-immunoreactive neurons were located using the allatotropin antiserum in a whole-mount immunofluorescence method adapted from Davis *et al.* (1989). Several cell types were found to be immunoreactive, including three pairs of intensely staining median neurons in abdominal ganglia 3–6 of the pharate adult. The location of these median pairs of cells in each ganglion is anterior, mid-dorsal and posterio-ventral (Fig. 1A). The cells project bilaterally *via* the ventral nerves of each ganglion to the next transverse nerve (Fig. 2), where their axons terminate in numerous superficial varicosities (Fig. 1D). It is well established that the transverse nerve is a neurohemal organ in Lepidoptera (Provansal, 1972; Taghert and Truman, 1982; Tublitz and Truman, 1985*a–d*). The location and projection pattern of these three pairs of median neurons indicate that they belong to a group of abdominal median neurosecretory cells identified previously (Taghert and Truman, 1982; Tublitz and Truman, 1985*a*). Using the nomenclature of Davis *et al.* (1993), these are the M_1, M_2 and M_5 neurosecretory cells. The M_1 and M_2 cells mature during adult development, but the M_5 cells are already differentiated in the larval stages (Taghert and Truman, 1982; Tublitz and Truman, 1985*a*). The M_5 cells of larvae were not stained by the allatotropin antiserum and only became immunoreactive, along with the M_1 and M_2 cells, during adult metamorphosis. Two pairs of the abdominal median neurosecretory cells were not allatotropin-immunoreactive: (1) the M_3 cells, which are ventral and mature during adult metamorphosis (Taghert and Truman, 1982); and (2) the M_4 cells, which are already differentiated in the larval stages (Tublitz and Truman, 1985*a*).

Previously, the abdominal median neurosecretory cells of adult *M. sexta* were reported

Key words: allatotropin, *Manduca sexta*, cardioacceleratory peptide.

Fig. 1. Whole mount of the abdominal ganglion (A–C) and transverse nerve (D–F) of adult *Manduca sexta* showing allatotropin- and CCAP-immunoreactive neurosecretory cells and processes in green and red respectively. Tissue was first processed for CCAP-immunoreactivity with a Rhodamine-labeled secondary antiserum and subsequently incubated with FITC-labeled anti-allatotropin IgG. The fluorescein-labeled allatotropin-immunoreactivity is visualized in A and D, the Rhodamine-labeled CCAP-immunoreactivity in B and E, and both types of immunoreactivity are visualized simultaneously in C and F. The median neurosecretory cells M_1, M_2, M_4 and M_5 and the lateral L_1 neurosecretory cells are indicated. Scale bars 50 μm (A,B,C), 125 μm (D,E) and 100 μm (F).

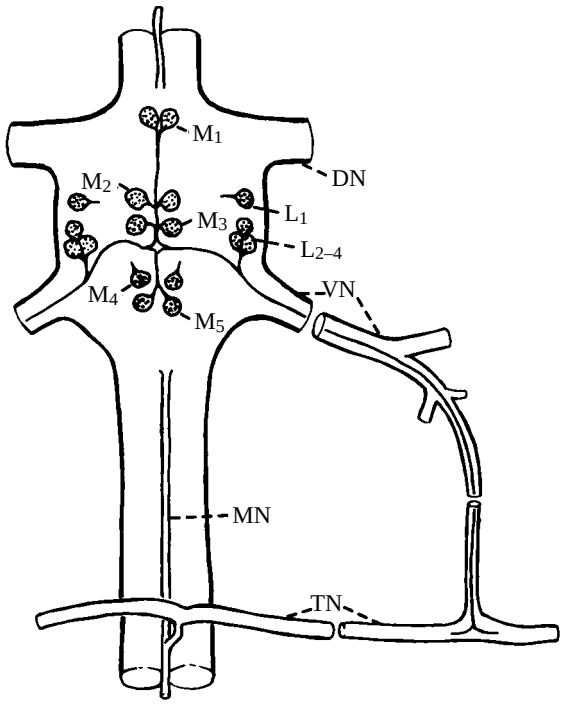
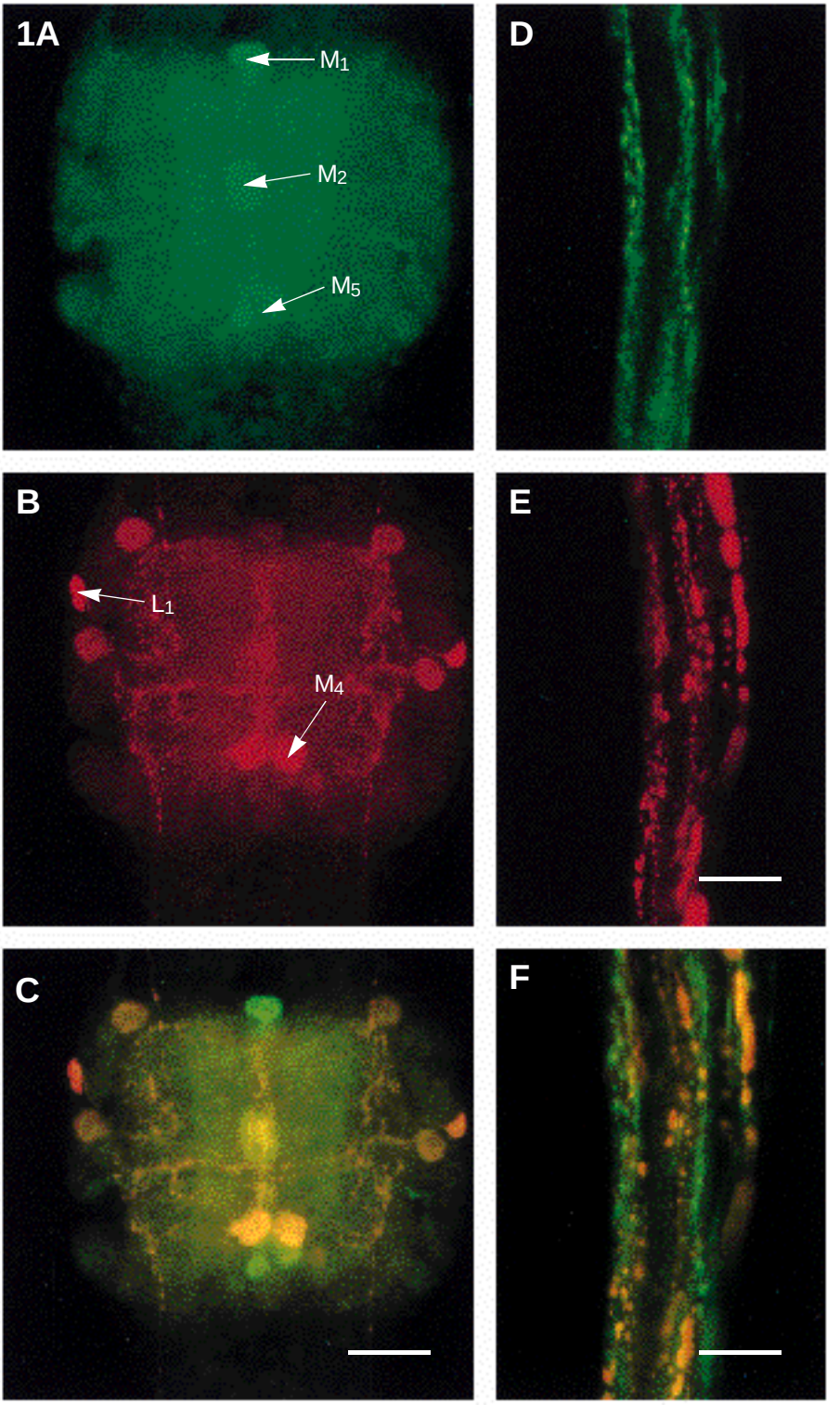


Fig. 2. Depiction of the neurosecretory cells of the fourth abdominal ganglion of *Manduca sexta* and of the projection of certain of these cells to the neurohemal transverse nerve *via* the ventral nerve. Cells L_1 and M_4 project to the transverse nerve *via* other pathways not shown. Neurosecretory cells M_1, M_2 and M_5 are allatotropin-immunoreactive, and previous studies have shown that cells L_1 and M_4 are immunoreactive to an antiserum to crustacean cardioactive peptide. (DN, dorsal segmental nerve; $L_{1–4}$, lateral neurosecretory cells of the abdominal ganglion; $M_{1–5}$, median neurosecretory cells of the abdominal ganglion; MN, median nerve; TN, transverse nerve; VN, ventral segmental nerve).

to contain two unidentified cardioactive peptides, CAP_1 and CAP_2 (Tublitz and Truman, 1985*a*,*b*); subsequently CAP_1 has been shown to consist of several peptides and CAP_2 of three peptides (Loi *et al.* 1992). One of the CAP_2 peptides has been identified as crustacean cardioactive peptide (CCAP), a peptide first identified from a shore crab by Stangier *et al.* (1987). This peptide has now been isolated and sequenced from *M. sexta* (Hildebrand *et al.* 1990; Cheung *et al.* 1992; Lehman *et al.* 1993). Moreover, CCAP has


1A
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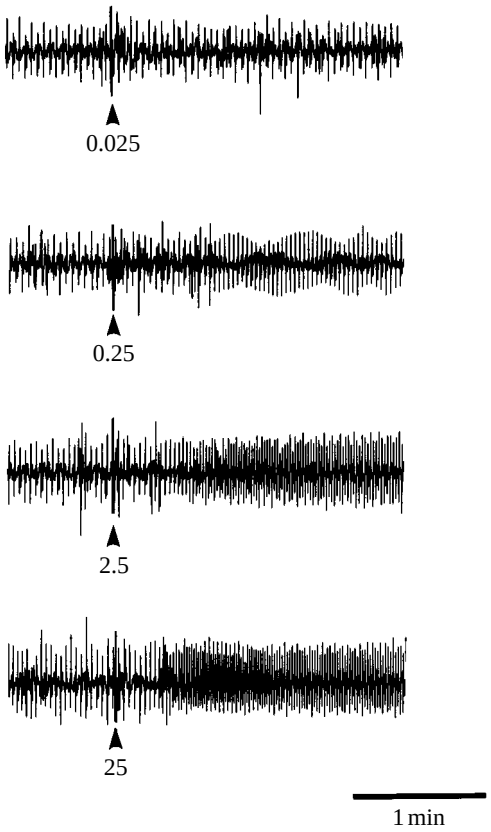

Fig. 3. Responses of the semi-isolated pharate adult heart of *Manduca sexta* to various concentrations of allatotropin. Peptides were added to the bath at the arrowheads; numbers indicate the number of picomoles added to the superfusion stream in a volume of 10 μl. The heart bioassay was performed as described by Lehman *et al.* (1993).

been shown to be cardioactive in *M. sexta* (Davis *et al.* 1990; Lehman *et al.* 1993). Using an antiserum that has been shown to be specific to CCAP (Dircksen and Keller, 1988; Stangier *et al.* 1988; Lehman *et al.* 1993), a pair of abdominal median neurosecretory cells, the M_4 cells, has been found to be CCAP-immunoreactive in ganglia of adults (Davis *et al.* 1993). These cells are not the same as the four pairs of so-called CAP cells identified by Tublitz and Sylwester (1990), using a monoclonal antibody (anti-CAP; Taghert *et al.* 1983, 1984) that has been reported to recognize an epitope common to CAP_1 and CAP_2.

For direct comparisons between CCAP- and allatotropin-immunoreactive neurosecretory cells in the same ganglia, double-fluorescence labeling was performed. Immunoreactivity to CCAP was visualized with a Rhodamine-labeled secondary antiserum (Fig. 1B), and the ganglia were subsequently incubated with purified allatotropin IgG, which had been labeled with FITC (Fig. 1A) using a kit from Boehringer Mannheim Biochemicals. The results clearly show that the two types of immunoreactivities are present in different cells (Fig. 1C). Moreover, the staining in the

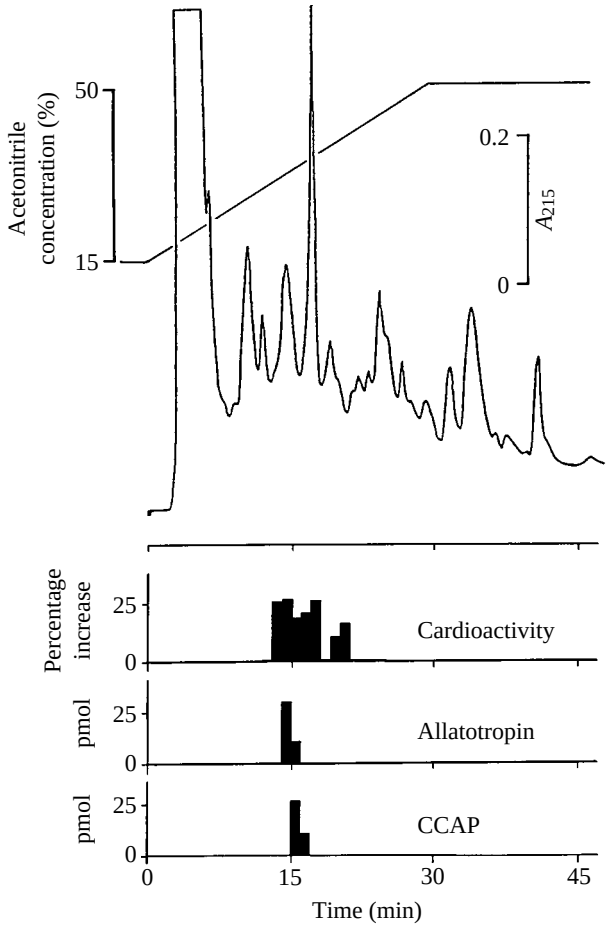

Fig. 4. HPLC chromatogram of 50 ventral nerve cords from *Manduca sexta*. Tissues were extracted and prepurified on a C-18 Sep-Pak as described by Veenstra and Hagedorn (1993). Separation was performed on an Alltech Versapack C-18 column, using 0.1 % trifluoroacetic acid (TFA) as the pairing ion and a gradient between 15 % and 50 % acetonitrile in water as indicated. Absorbance was measured at 214 nm and 1 ml fractions were collected. Samples of each fraction were tested on the heart of a pharate adult of *Manduca sexta* and assayed for allatotropin-immunoreactivity (Veenstra and Hagedorn, 1993) and CCAP-immunoreactivity (Lehman *et al.* 1993) by ELISA. Results are expressed as the percentage increase over the basal heart rate obtained with 0.25 abdominal nerve cord equivalents or pmol peptide per fraction. Previous studies have shown that the allatotropin-immunoreactive material in the abdominal ganglia co-elutes on HPLC with synthetic allatotropin (Veenstra and Hagedorn, 1993) and that the CCAP-immunoreactive peptide is CCAP (Lehman *et al.* 1993).

CCAP- and allatotropin-immunoreactive processes in the transverse nerve does not overlap (Fig. 1D–F).

Other than CCAP, the identities of the cardioactive peptides of *M. sexta* remain unknown, but the staining of median neurosecretory cells by allatotropin antiserum leads to the hypothesis that one of these peptides is allatotropin. When allatotropin was tested on the heart of pharate adult (stage 18) *M. sexta*, the peptide was strongly excitatory

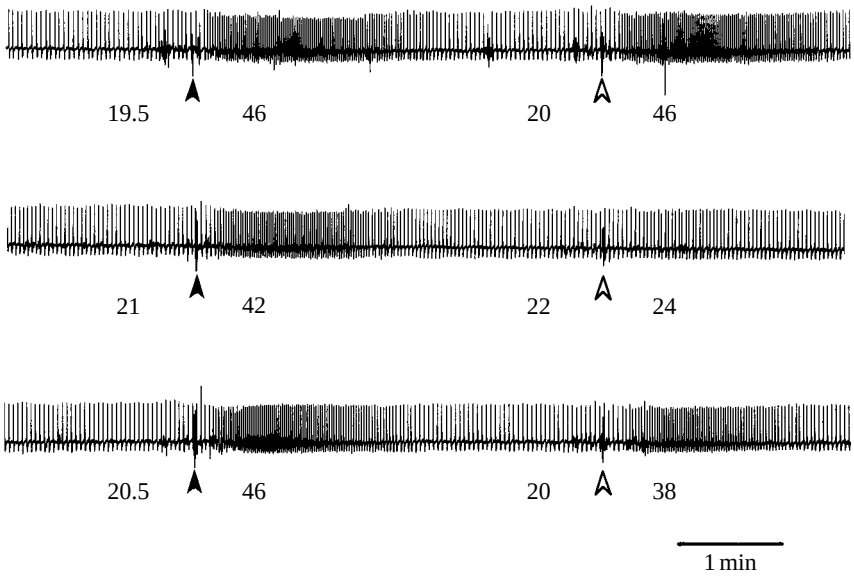

Fig. 5. Cardioexcitatory effects of HPLC fractions 14, 15 and 16 (0.25 abdominal nerve cord equivalents) on the heart of the pharate adult *Manduca sexta* and the interference with these effects by allatotropin antiserum. Upper trace: effects of fraction 14 added at the filled arrowhead and effects of fraction 14 preincubated with anti-allatotropin added at the open arrowhead. Middle trace: effects of fraction 15 added at the filled arrowhead and the effects of fraction 15 preincubated with anti-allatotropin added at the open arrowhead. Lower trace: effects of fraction 16 added at the filled arrowhead and the effects of fraction 16 preincubated with anti-allatotropin added at the open arrowhead. Numbers below the traces are heart rate (beats min^{-1}) before and after application of peptide. The amounts of immunoreactive allatotropin in fractions 14, 15 and 16 were 0, 30 and 11 pmol respectively. Samples of HPLC fractions were lyophilized with 100 μg of radioimmunoassay-grade bovine serum albumin and dissolved in either saline or antiserum diluted (diluted 1:20) in saline. They were then left for 1 h at room temperature before being assayed.

(Fig. 3). To provide further evidence in support of our hypothesis, an extract of abdominal nerve cords was separated on HPLC, and the fractions containing allatotropin-immunoreactivity, CCAP-immunoreactivity and those that had an excitatory effect on heart preparations were identified.

Two regions eliciting cardioexcitatory activity were found: these were in fractions 14–18 and in fractions 20–21. Allatotropin-immunoreactivity was identified in fractions 15 and 16, while fractions 16 and 17 exhibited CCAP-immunoreactivity (Fig. 4). Preincubations of the cardioactive fractions with allatotropin antiserum (1:20) abolished all of the cardioacceleratory activity present in fraction 15 and some of the effect of fraction 16, but such treatment was without effect on the cardioacceleratory activity of fractions 14, 17, 18, 20 and 21 (Fig. 5). The presence of both allatotropin and CCAP-immunoreactivity in fraction 16 suggested that the cardioactivity remaining in fraction 16 after preadsorption with allatotropin antiserum might be due to CCAP. Preincubation of fraction 16 with both anti-allatotropin and anti-CCAP abolished all cardioactive effect,

but preincubation with anti-CCAP alone only reduced the cardioactive effect of this fraction. Preincubation with CCAP antiserum abolished the cardioacceleratory activity of fraction 17, but was without effect on fractions 14, 15, 18, 20 or 21.

Previously, the anti-CAP antibody has been shown to block the cardioacceleratory effects of CAP_1 and CAP_2 (Tublitz and Evans, 1986). It is now known that the so-called CAPs comprise at least five cardioactive peptides (Loi *et al.* 1992), and this conclusion is supported by the present study. Therefore, the anti-allatotropin and anti-CCAP antisera are apparently more specific in their blocking of the cardioacceleratory effects of the CAPs than is the anti-CAP monoclonal antibody. This greater specificity is also seen in the use of these antisera in immunocytochemistry; the CCAP (Davis *et al.* 1993) and allatotropin antisera recognize subpopulations of the abdominal neurosecretory cells (Fig. 1C), while the anti-CAP monoclonal antibody recognizes all but one pair of these cells (Tublitz and Sylwester, 1990).

The apparently different separation characteristics of our C-18 column, compared with the one used by Tublitz (e.g. Tublitz, 1989), make it difficult to determine whether any of the previously isolated CAP fractions may be the same as allatotropin. However, because the relative molecular mass of CAP_1 was estimated to be about 1000, and that of CAP_2 about 500 (Tublitz and Truman, 1985*a*), it seems more likely that one of the peptides in the CAP_1 fraction is allatotropin (M_r 1487). This conclusion is supported by the report that CAP_1 was not found in larval abdominal ganglia (Tublitz and Sylwester, 1990), and, correspondingly, we found no allatotropin-immunoreactive neurosecretory cells in larval ganglia.

Another characteristic of CAP_1 is that it does not stimulate the heart rate in larvae (Tublitz *et al.* 1992). Therefore, we tested the effects of allatotropin and CCAP on the larval heart. The effects of CCAP were in marked contrast to those of allatotropin. Whereas 50 pmol of allatotropin failed to produce an observable change in heart rate, an equal amount of CCAP produced a maximal response on the larval heart (N=4). This amount of CCAP or allatotropin was sufficient to produce maximal responses in the pharate adult heart (Figs 1, 5). These findings, taken together, suggest that one of the peptides present in CAP_1 is indeed allatotropin.

It has been shown before that both CAP_1 and CAP_2 are released at the time of eclosion and later, during flight (Tublitz and Truman, 1985*c*; Tublitz and Evans, 1986; Tublitz, 1989). Therefore, it seems likely that allatotropin is also released at these times, but, because there is more than one peptide in the CAP_1 fraction (Loi *et al.* 1992), it cannot be assumed that allatotropin is released. We were able to measure allatotropin-immunoreactive material in Sep-Pak prepurified hemolymph samples obtained at eclosion, but the immunoreactive material did not co-elute with authentic allatotropin on HPLC. Hence, the release of allatotropin after eclosion still remains to be demonstrated.

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