Allatotropin released by the brain controls larval molting in *Galleria mellonella* by affecting juvenile hormone synthesis

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ABSTRACT Monoclonal antibodies were raised against a protein fraction which was purified from larval brains of *Galleria mellonella* and which was shown to stimulate the *corpora allata* to synthesize juvenile hormone *in vivo* as well as *in vitro*. Immunoblot analysis revealed that one polypeptide band of 20 kDa was specifically recognized. Two pairs of median neurosecretory cells of the brain and the cells of the *corpus cardiacum* were demonstrated to be immunoreactive to the antibodies. Our results sustain the hypothesis that the larval brain directly governs insect larval molting by controlling JH synthesis via an allatrophic factor.

KEY WORDS: *Galleria mellonella, juvenile hormone, allatotropin, monoclonal antibodies, neurosecretory cells*

Introduction

The development of holometabolous insects is under the control of two hormone classes, ecdysteroids and juvenile hormones (JH). Precisely timed fluctuations in the titre of the morphogenetic hormones are critical for normal larval development and metamorphosis. While high titres of 20-hydroxy-ecdysone initiate each molt, the type of molt completed is essentially determined by the concentration of JH. During larval-to-larval molts high JH titres are required whereas the decline of JH during the final larval instar is necessary for metamorphosis to occur (for review, see Riddiford, 1994). In adults JH stimulates reproductive maturation (Koeppe *et al.*, 1985).

The regulation of ecdysteroid and JH production is best understood in lepidopteran insects, in which the major controlling factors are the adenotropic neuropeptides or polypeptides produced by specific small groups of neurosecretory cells in the brain. In response to external and internal signals the brain releases the prothoracicotropic hormone (PTTH) which stimulates the prothoracic glands to synthesize ecdysone.

JH is synthesized and released by the *corpora allata* (CA) which are tightly bound to the neurohemal organ, the *corpora cardiaca* (CC), in lepidopteran insects. The glands are connected with the brain by nerve fibres composed of axons of cerebral neurons and neurosecretory cells (Matsumoto and Ishii, 1992). Environmental and physiological cues are integrated by the brain which controls the CA by stimulatory or inhibitory factors directly via nervous connections or indirectly via hemolymph circulation. Neuropeptides that either stimulate (ATTH, "allatotropin") or inhibit ("allatostatin", "allatoinhibin") the CA to produce JH originate in the brain (Granger *et al.*, 1984; Sehnal and Rembold, 1985; Bhaskaran *et al.*, 1990; Bogus and Scheller, 1994). A scheme for the hormonal control of the postembryonic development of *Galleria mellonella* is depicted in Figure 1. Though some aspects of these events are still hypothetical, the assumptions are supported by a growing body of evidence derived from a variety of studies.

Since no larval allatotropin was characterized and no allatotropin synthesizing cells were identified in any insect larva, we have focused our efforts on (i) the partial purification of an allatotropic factor from larval brains of *Galleria mellonella*, and (ii) the immunolocalization of neurosecretory cells in the brain using monoclonal antibodies.

Effect of juvenilizing treatments on the titre and biosynthesis of JH

Under favorable conditions the larval development of *Galleria mellonella* consists of 6 instars lasting 2-4 days each and a last (= 7th) instar lasting 8 days. The genetically fixed program can be disturbed by overcrowding, injury, inadequate food and space supply, or low rearing temperature, all causing a delay in metamorphosis (Sehnal, 1966; Pipa, 1971; Bogus *et al.*, 1986; Bogus and Wolbert, 1987; Smietanko *et al.*, 1989). However, juvenilizing treatments like cooling, brain implantation or application of juvenile hormone analogues, when applied at the beginning of the final instar, generate molting into a supernumerary (= 8th) larval molt (Sehnal and Meyer, 1968; Sehnal and Granger, 1975; Cymborowski and Bogus, 1976). Extra-larval molts can be induced in several lepidopteran insects, however, *Galleria* is the favorable experimental system as this phenomenon can be provoked in nearly 100% of the larvae tested. It has been shown that the juvenilizing treatments affect JH synthesis resulting in an increase in the hormone titre (Bogus and Cymborowski, 1981, 1984; Sehnal and Rembold, 1985; Bogus and Scheller, 1991a). We have shown that short time exposition (3h) of last instar

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Galleria larvae to 0°C resulted in supernumerary molts which are closely related with a strong increase in the JH titre due to an increased JH synthesis rate of the CA. One day after chilling the level of JH was about 18,000 "*Galleria* Units" (GU) per gram of tissue compared to 75 GU/g before cooling (Bogus and Scheller, 1988; Scheller and Bogus, 1988).

JH titres were measured in whole body homogenates using the "*Galleria* wax wound test" (de Wilde *et al.*, 1968; Hsiao and Hsiao, 1977).

Correlation between JH synthesis and allatotropic activity

The aim of our experiments was to give direct evidence that larval brains of *Galleria* control the biosynthetic activity of the CA. The allatotropic activity of larval brains was measured by their capability of stimulating JH synthesis in isolated CA.

To determine the rates of JH biosynthesis we applied an *in vitro* radiochemical assay which uses the incorporation of the methyl moiety of L-[H3-methyl]-methionine into JH. The newly synthesized JH was quantified by a partition assay (Feyereisen and Tobe, 1981).

Since the *corpora allata* of *Galleria* are intimately associated with the *corpora cardiaca* it is not possible to prepare CA without contaminant CC. Therefore, one CA-CC pair was incubated for 3 h at 30°C in 20 µl TC199 medium (Gibco), pH 7, with 1 µCi L-[H3-methyl]-methionine (83 Ci/mmol) together with one brain dissected from chilled larvae 24 h after termination of the cooling stress when the allatotropic activity of the brain is at a maximum.

Our data (Bogus and Scheller, 1991a) demonstrate that co-incubation of CA-CC with brains from previously chilled larvae resulted in an 2 to 3-fold increase in JH synthesis (156±71 pmole JH per CA-CC during 1 h) compared to the controls (32±6 pmol JH). Analyses of the incubation products by thin layer chromatography with 3 different solvent systems showed that no other product besides JH became radioactively labeled. Gas liquid chromatography revealed that the synthesis product is JH II which is known to represent the main sesquiterpenoid in lepidopteran larvae. JH II is the only JH circulating in *Galleria mellonella* larvae (Sehnal and Rembold, 1985). Furthermore, it has been demonstrated that isolated CC-CA complexes from *Galleria* last instar larvae produce only JH II (Dahm *et al.*, 1976).

From the above results the question arises, which kind of factor(s) released by the brain is responsible for the stimulation of the CA to synthesize JH. That is why we fractionated the culture media after incubation with brains of chilled larvae by ion exchange chromatography. The allatotropic activity of each fraction was tested *in vitro* as well as *in vivo*.

Separation of brain proteins and identification of an allatotropic factor

10 brains from chilled larvae were incubated in 200 µl sterile tissue culture medium TC-199 (Gibco-BRL) for 3 h at 30°C with gentle mixing. After termination of the incubation the brains were removed by centrifugation and the supernatants were frozen at -20°C.

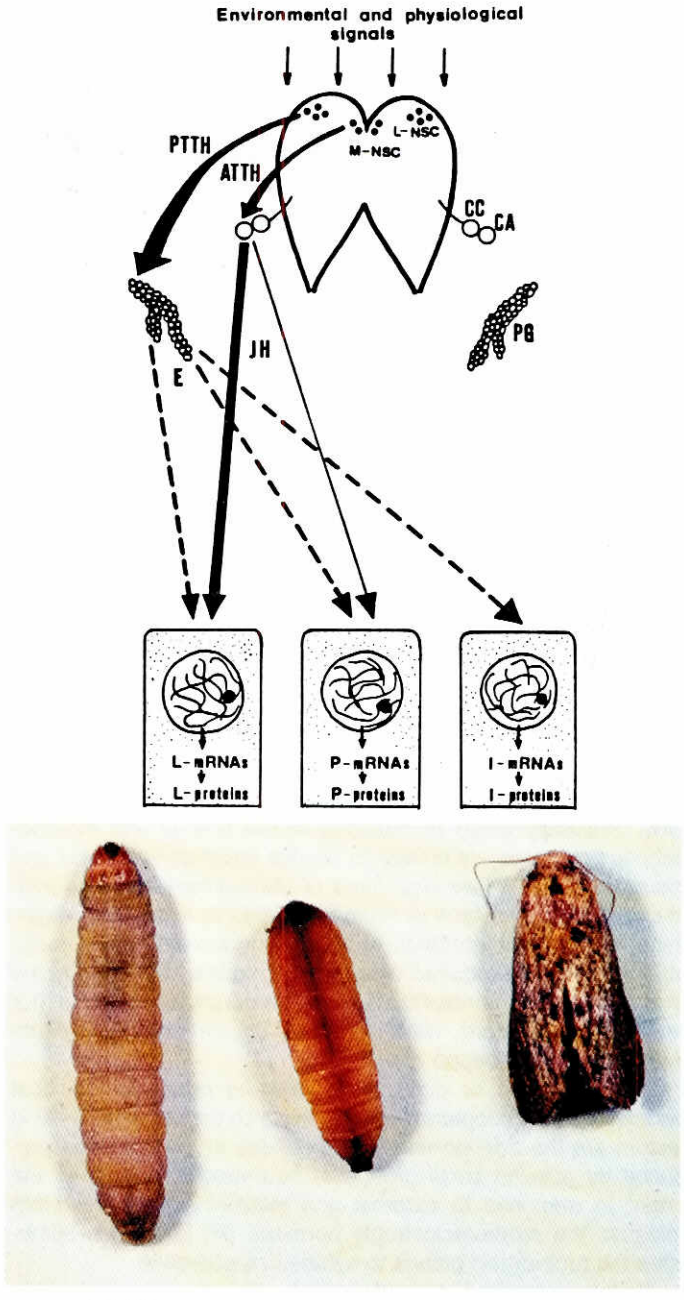

Fig. 1. Scheme for the hormonal control for the postembryonic development of *Galleria mellonella*. *CA, corporus allatum; CC, corpus cardiacum; PG, prothoracic gland; E, ecdysone; JH, juvenile hormone; ATTH, allatotropic hormone; PTTH, prothoracotropic hormone; L-NSC, lateral neurosecretory cells; M-NS, medial neurosecretory cells.*

The supernatants of 148 samples (1480 brains incubated) were pooled, dialyzed against water and lyophilized. The proteins were dissolved in 20 mM TRIS-HCl, pH 7, applied to a FPLC ion-exchange column Mono Q (Pharmacia) and eluted by a linear 0 - 1 M NaCl gradient in 20 mM TRIS. The elution rate was 1 ml/min. We employed the *in vitro* radiochemical method as described above for assaying the ability of each

fraction to stimulate isolated CC-CA complexes to synthesize JH II. For that purpose the 2 ml-fractions were dialyzed against water, lyophilized and dissolved in 100 µl water. 1 µl was added to the incubation medium containing 1 pair of CC-CA complex. Ten independent determinations were executed for each fraction.

One fraction (AF) eluted by 0.20 M NaCl (Fig. 2) increased the rate of JH synthesis about 7-fold from 100 to 690 pmol JH II per hour and gland pair and was therefore considered to be an allatotropic factor. This result was sustained by the fact that more than 50% of the larvae underwent a supernumerary molt after injection with 5 µg AF-protein. JH synthesis caused by all other fractions from the Mono-Q column did not differ from the basal rate in the range of 50-140 pmol and were not able to induce extra-larval molts.

SDS-PAGE of *in vitro* [H3]-labeled AF revealed a single polypeptide band with an apparent molecular mass of 20 kDa. This polypeptide accumulated in the culture medium during 3 h incubation of brains from chilled larvae (Fig. 3).

From our work it appears that the concentration of the 20 kDa polypeptide increases 10 to 20-fold after chilling stress. When the JH analogue hydropren was topically applied to last instar larvae a decrease in the allatotropic activity of the brain, accompanied by the disappearance of the 20 kDa protein, was detected (Bogus and Scheller, 1991b). Taking all these evidences together it has become indicative that the 20 kDa protein exhibits a larval allatotropin. There was no report to date on the purification and molecular characterization of a larval allatotropin with a stimulating effect on JH synthesis. *In vitro* stimulation of the larval CA by the brain of *Manduca sexta* has been documented (Granger *et al.*, 1984). Allatotropic fractions extracted from brains of pupae (Granger *et al.*, 1984) or pharate adults of *Manduca* (Kataoka *et al.*, 1989) and from brains and CC of adult *Locusta migratoria* (Ferenz and Diehl, 1983; Rembold *et al.*, 1986) exhibit a high diversity ranging from 700 Da to 40 kDa. A 13-residue neuropeptide purified from head extracts of pharate adult *Manduca* had no discernible effect on the rate of JH biosynthesis of larvae or pupae (Schooley *et al.*, 1990). It has been concluded from the authors that the activity of *Manduca sexta* allatotropin may be restricted to the adult stage of the order Lepidoptera.

TABLE 1

INHIBITION OF *IN VITRO* JH SYNTHESIS BY 4 MONOCLONAL ANTIBODIES AGAINST THE ALLATOTROPIC FACTOR AF

Hybridoma Fraction #	CA-CC (n replicates)	*In vitro* JH synthesis pmol/h x CA-CC	% inhibition
–	5	120+30*	–
–	15	280+50**	–
AF3	9	100+20	64%
AF43	9	70+20	75%
AF137	8	130+10	54%
AF139	6	70+10	75%

* Basal JH synthesis of unstimulated glands which were incubated without brains. The percentage of inhibition correspond to the synthesis rate of stimulated glands without antibody (**). ± standard error of the means. The fractions AF3, AF137, and AF139 were identified by ELISA as IgG types and AF43 as a IgM type.

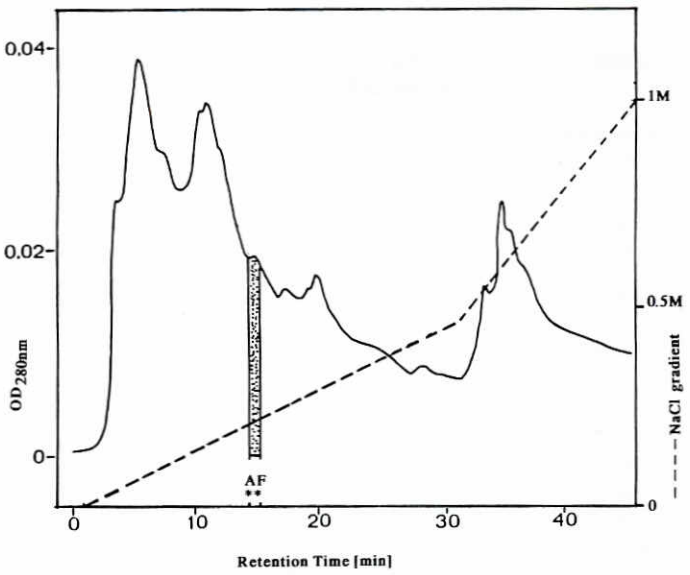

Fig. 2. Isolation of a protein with allatotropic activity from larval *Galleria* brains. *Proteins released by 1480 brains into the incubation medium were separated by Mono Q-FPLC (Bogus and Scheller, 1994).*

Monoclonal antibodies against an allatotropic factor

Monoclonal antibodies were obtained after immunizing BALB/c mice with the allatotropic fraction AF. Mice were immunized intraperitoneally with 250 µl of complete Freud's adjuvant plus AF (50 µg protein) followed by three booster injections with incomplete adjuvant containing AF over a period of 2 months. Fusing was performed according to Köhler and Milstein (1975). We obtained about 200 hybridoma colonies from which 33 were chosen for our further investigations. The hybridoma supernatants were dialyzed against water, lyophilized and then tested for their ability to inhibit the stimulation of JH synthesis *in vitro*. 100 µg protein from each hybridoma supernatant was co-incubated for 1h at 30°C with 1 CC-CA pair from a normally grown 1d-LL and 1 highly active brain dissected from a chilled larva.

Four hybridoma supernatants were found to inhibit the enhanced JH-synthesis of CA-CC *in vitro* (Table 1). Denatured antibodies (boiled for 5 min) as well as bovine serum albumin or anti-arylphorin IgGs from *Calliphora* larvae did not show any inhibition. The same hybridoma clones were applied for an *in vivo* test: Chilled last instar larvae received injections of the 4 hybridoma supernatants (50 µg protein in 5 µl volume) and the ability of the antibodies to prevent extra-larval molts was checked. From Table 2 it can be seen that those antibodies which depress JH synthesis *in vitro* suppress chilling-stimulated molts as well.

Immunoblot analysis

Brains were dissected from chilled last instar larvae and separated by one-dimensional SDS-PAGE (Laemmli, 1970). The proteins were renatured by incubating the gels in buffer R (10 mM TRIS-HCl, pH 7.5, 4 M urea, 2 mM EDTA, 50 mM NaCl, 0.1 mM dithiothreitol) for 2 h at room temperature without moving. Afterwards the gels were soaked for 15 min in 25 mM TRIS-

TABLE 2

INHIBITORY EFFECT OF 4 HYBRIDOMA SUPERNATANTS ON EXTRA-LARVAL MOLTS

Hybridoma Fraction #	chilled larvae (n)	extra-larval molts (N)	% inhibition of N
–	43	43	0%
AF3	45	20	55%
AF43	44	22	50%
AF137	46	24	47%
AF139	46	24	47%

The contol larvae received injections of 5 µl insect saline

HCl, pH 9.4, 40 mM norleucine and then transferred electrophoretically to nitrocellulose membranes Polytrap 296 PE (Schleicher and Schuell) at 200 mA for 2 h. The NC membranes were presoaked for 10 min in 25 mM TRIS-HCl, pH 10.4. After the electrotransfer the filters were incubated in blocking solution TTBS (10 mM TRIS-HCl, pH 7.5, 0.9% NaCl, 0.3% TWEEN 20) overnight at 4°C. After incubation with non-diluted hybridoma supernatants at room temperature for 2 h the filters were rinsed for 15 min with TTBS and subsequently incubated with the second antibody, biotinylated goat anti-mouse IgG, following the instructions of the supplier (Vectastain kit, Vector Laboratories).

When protein homogenates of brains from chilled larvae were separated by one-dimensional SDS-PAGE and examined by immunoblotting we observed that one protein band with an apparent molecular mass of 20 kDa could be recognized by all 4 hybridoma supernatants tested. (AF 3, AF 43, AF 137, and AF 139).

Identification of neurosecretory cells by immunostaining

Whole-mounts

About 20 brains were incubated in 1.6 ml 0.1M HEPES, 2 mM $MgSO_4$, 1 mM EGTA, pH 6.9, containing 0.4 ml 20% paraformaldehyde and 8 ml benzine for 2 h at 37°C under vigorous shaking. The benzine phase was removed and 10 ml methanol p.a. was added. After 2 h incubation time the methanol was exhausted and solution ME (90% methanol, 0.05 mM EGTA) was added. The brains were then refixed and dehydrated by passage through a series of steps consisting of ME/PP (PP= 4% paraformaldehyde in PBS). The first step was a 5 min incubation in ME/PP mixture 7:3, the second 5 min in ME/PP 1:1, the third 5 min in ME/PP 3:7, and the last step 20 min in PP. The brains were washed in PBS for 10 min and directly subjected to immunostaining. The fixed brains were preincubated for 2 h with horse serum, subsequently incubated over night in non-diluted hybridoma supernatant at 4°C and immunostained using the horseradish peroxidase "ABC Vectastain kit" (Vector Laboratories). For immunochemistry we used the monoclonals AF3, AF137, AF139.

Semi-thin sections

Brains of chilled larvae were fixed for 2 h in 4% paraformaldehyde, then kept overnight in *Drosophila* Ringer and sliced into 10 µm cryosections. Immunostaining was performed using an "ABC kit" applying biotinylated avidin and horseradish peroxidase following the protocol provided by the supplier. The hybridoma supernatants were diluted 1:2 with PBS containing 0.1% TRITON X-100.

When monoclonal antibodies were applied to whole-mounts of brains from chilled last instar larvae immunocytochemistry revealed the selective staining of two cells located in the *pars intercerebralis* of the brain and in the *corpus cardiacum* (Fig. 4B). Inspection of the cryosections showed that two pairs of cells in the *pars intercerebralis* were immunoreactive. Cells in the corpus cardiacum were also stained in contrast to the *corpus allatum* where no staining was visible (Fig. 4C,D). The stained median neurosecretory cells have been implicated in the control of the CA. They show the ability to elicit supernumerary larval molts after implantation into sensitive host larvae and the ability to stimulate JH synthesis *in vitro* (Muszynska-Pytel, 1987). When those cells are removed the larval *Galleria* brain looses its allatotropic activity (Granger and Sehnal, 1974).

The results above, summarizing our work on allatotropin, sustain the hypothesis that the larval brain of *Galleria mellonella* directly controls JH synthesis by stimulating the biosynthetic activity of the CA by a neurohormone.

The specific immunostaining of the CC may reflect the storage function of this neurohemal organ. Although we were not able to convincingly show immunostaining within the axons we suggest that allatotropin which is produced in neurosecretory cells of the *pars intercerebralis* is transported via axon into the CC where it accumulates. In the present stage of our studies we cannot exclude that neurosecretory cells in the CC synthesize allatotropin too and we are not able to explain how allatotropin is transferred from CC to the target cells in the CA. This question could be answered by *in situ* hybridization using the allatotropin-cDNA clones as a probe.

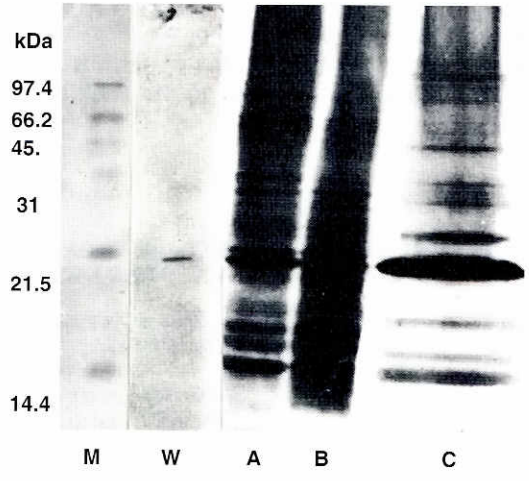

Fig. 3. SDA-PAGE of proteins from chilled *Galleria* last instar larvae. **(A-C)** *Silver staining of proteins from 20 larvae* **(A)** *before and* **(B)** *after 3 h incubation in TC-199 medium.* **(C)** *Polypeptides accumulated during incubation in the medium.* **(W)** *Western blot: Immunostaining with allatotropin monoclonal antibody AF 139 (The antibodies AF3, AF43, and AF137 specifically recognized the same band).* **(M)** *Molecular weight marker.*

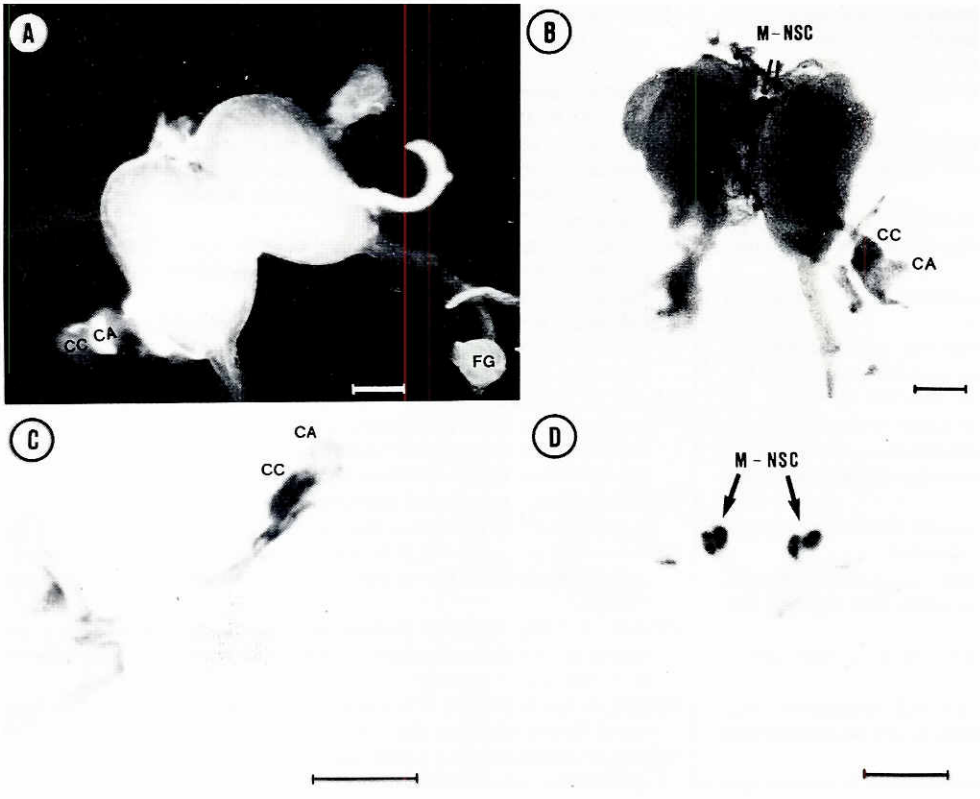

Fig 4. Immunohistochemical localization of allatotropin containing cells. **(A)** *Unstained brain.* **(B)** *Whole-mount immunostaining.* **(C,D)** *Immunostaining of semithin cryo-sections. CA, corpus allatum; CC, corpus cardiacum; M-NSC, median neurosecretory cells; FG, frontal ganglion. Bars, 0.1 mm.*

We have established a cDNA library from poly(A)$^+$RNA isolated from brains of chilled larvae. Screening the library with the 4 antibodies mentioned above resulted in 2 positive clones. However, we have not succeeded in isolating a full length transcript until yet. From comparison of our preliminary sequencing data with other sequences published we found some analogies to the neurohormone bombyxin which displays a group of polypeptides (about 5 kDa) with PTTH-like activity and which is structurally homologous to insulin. The neurosecretory cells of *Galleria* specifically stained with AF3, AF137, and AF139 also show bombyxin-like immunoreactivity (Zitnan *et al.*, 1990). Furthermore, it has been shown that superior median protocerebrum cells of *Drosophila* showed immunoreactivity with antibodies raised against allatotropin of adult *Manduca sexta* (Zitnan *et al.*, 1993). Taking together all these results and the fact that the same neurosecretory cells are immunostained in many cases by a variety of antibodies raised against different insect neurohormones one might speculate that allatotropin as well as other neurosecretory molecules bear common epitopes and generate from a preprohormone which is spliced into the different neurosecretes. This last suggestion can be sustained by our observation that the 20 kDa allatotropin is not synthesized *de novo* in the *Galleria* brain after treatments enhancing the allatotropic activity, although it accumulates (Bogus and Scheller, 1991b).

Our working hypothesis that different brain hormones with different modes of action derive from a common gene and are spliced into their active forms by posttranscriptional or post-translational processes can be confirmed or refuted when the structures and sequences of the corresponding genes and mRNAs are elucidated. This type of work is underway in our laboratory.

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