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Immunocytochemical localization of an allatotropin in developmental stages of *Heliothis virescens* and *Apis mellifera*

Julie M. Glasscock[a], Akira Mizoguchi[b], Anna Rachinsky[a,*]

[a]*Department of Biology, University of Minnesota Duluth, 211 Life Science Building, 1110 Kirby Drive, Duluth, MN 55812, USA*
[b]*Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan*

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Abstract

Juvenile hormone biosynthesis by the corpora allata is regulated by stimulatory neuropeptides called allatotropins and inhibitory neuropeptides called allatostatins. This study localized *Manduca sexta* allatotropin-like material in developmental stages of the noctuid moth *Heliothis virescens* and the honeybee *Apis mellifera*. Immunocytochemical methods using both fluorescence-tagged antibodies and enzyme-coupled antibodies were used to stain the central nervous tissue of both species. *H. virescens* contains *M. sexta* allatotropin (Manse-AT)-like material consistently throughout larval development. The distribution patterns of Manse-AT immunoreactive cell bodies in the CNS persisted from one larval instar to the next. It will be discussed how larval Manse-AT distribution patterns differed from those in adults. The total number of AT-containing cells in brain and subesophageal ganglion gradually increased during larval development, whereas in the thoracic and abdominal ganglia, the number of AT-containing neurons remained constant. In the honeybee *A. mellifera,* Manse-AT immunoreactive cells were only found in a few brains from late last instar larvae (prepupae). Manse-AT-like material was present in a group of 6–8 cells in the pars intercerebralis. However, we did not find any Manse-AT-like material in brains of early last instar larvae, whose corpora allata (CA) are more sensitive to in vitro stimulation by Manse-AT than prepupal CA.

Keywords: Juvenile hormone biosynthesis; Corpora allata; Neuropeptides; Allatotropin; *Heliothis virescens*; *Apis mellifera*; Caste development

1. Introduction

Juvenile hormone (JH) is an important morphogenetic hormone that regulates development and reproduction in insects. Its most conserved function in regulating development is to delay the onset of metamorphosis in larval stages (Riddiford, 1994). Several more specific functions of JH have also been recorded, such as controlling caste differentiation in the honeybee, *Apis mellifera* (Nijhout and Wheeler, 1982), and migration in a variety of species (Rankin et al., 1986; McNeil and Tobe, 2001). Synthesis and release of the hormone occurs in the corpora allata (CA), a pair of neurohemal endocrine glands. JH titer is controlled by both regulation of CA activity (Feyereisen, 1985; Tobe and Stay, 1985) and also by catabolic enzymes in the hemolymph (Hammock, 1985). CA activity regulation has been attributed to several classes of allatoactive peptides, namely the stimulatory neuropeptides, allatotropins (AT), and inhibitory neuropeptides, allatostatins (Stay and Woodhead, 1993; Stay et al., 1994; Stay, 2000). *Manduca sexta* allatotropin (Manse-AT) is an amidated tridecapeptide that was originally isolated from brains of pharate adult insects, and has been shown to stimulate the corpora allata to produce JH at elevated rates in adults (Kataoka et al., 1989). Synthetic Manse-AT stimulated adult CA in vitro in several lepidopteran species (*Lacanobia oleracea*, Audsley et al.,

*Corresponding author. Tel.: +1 218 726 7270; fax: +1 218 726 8142.
E-mail address: arachins@d.umn.edu (A. Rachinsky).

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1999; *Spodoptera frugiperda,* Oeh et al., 2000; *Pseudaletia unipuncta*, Koladich et al., 2002), including the tobacco budworm, *Heliothis virescens* (Teal, 2002; Rachinsky et al., 2003). This correlates well with the detection of Manse-AT immunoreactivity in corpora cardiaca/corpora allata (CC/CA) complexes of adult *L. oleracea*, *M. sexta and H. virescens* (Duve et al., 2003; Rachinsky, unpublished results). Adults of *H. virescens*, like the adults of several other lepidopteran species (Truesdell et al., 2000; Tu et al., 2001) contain Manse-AT-immunoreactive neurons in all regions of their central nervous system and in their stomatogastric nervous system (Rachinsky, unpublished results). The distribution of Manse-AT containing cells in adult *H. virescens* brains has been resolved to the dorso-lateral and the posterior-lateral regions of the protocerebrum, and the base of the antennal lobe.

Considerably less is known about function, cellular distribution and timing of expression of Manse-AT in developmental stages of *H. virescens*. Manse-AT immunoreactivity was found in the frontal ganglion of *L. oleracea*, *M. sexta and H. virescens larvae* (Duve et al., 1999; 2000; Duve and Thorpe, 2003), but was not present in larval CC/CA complexes (Duve et al., 2003). Synthetic Manse-AT has been shown to stimulate larval CA in vitro in the tomato moth, *L. oleracea* (Audsley et al., 2000), and in a non-lepidopteran species, the honeybee, *A. mellifera* (Rachinsky et al., 2000), indicating that Manse-AT might be involved in regulating CA activity during insect development as well. This is supported by the presence of Manse-AT-like material in the larval central nervous system (CNS) of *M. sexta* and *L. oleracea* (Veenstra and Hagedorn, 1993; Taylor et al., 1996; Bhatt and Horodyski, 1999; Audsley et al., 2000). In addition, expression of the Manse-AT gene has been documented in the CNS of larval stages of *M. sexta* and *S. frugiperda* (Taylor et al., 1996; Bhatt and Horodyski, 1999; Abdel-latief et al., 2003, 2004a). We are interested in knowing if Manse-AT is present in the developmental stages of *H. virescens* because it is unknown if this peptide contributes to regulation of JH biosynthesis during this time. Knowing the presence and location of Manse-AT containing cells would suggest possible functions of the peptide during development, including CA stimulation.

This study also used immunocytochemical methods to localize Manse-AT containing neurons in selected developmental stages of the honeybee, *A. mellifera*. The honeybee is one of a few non-lepidopteran species in which Manse-AT has been shown to stimulate the CA in vitro (Rachinsky and Feldlaufer, 2000; Tu et al., 2001). During the last larval instar, the two female honeybee castes, queens and workers, display considerable stage- and caste-specific differences in JH biosynthesis rates and JH titer (Rachinsky and Hartfelder, 1990; Rachinsky et al., 1990), which indicate that JH may be a key regulator of queen development in this species. In queen larvae, JH biosynthesis peaks during the feeding stage of the last instar, then decreases during the early spinning stage and shows a second, lower peak early in the prepupal stage, before ceasing right before the pupal molt. In worker larvae, JH biosynthesis is low throughout the feeding and spinning stages of the last instar, then rises to a shallow peak at the beginning of the prepupal stage before ceasing right before the pupal molt. Since Manse-AT is capable of stimulating the CA of worker larvae in vitro (Rachinsky et al., 2000), and larval worker brains contain some yet unidentified allatotropic factor (Rachinsky, 1996), we were interested in finding out if Manse-AT-like material was present in developmental stages of the honeybee.

2. Material and methods

2.1. Insects

H. virescens larvae were obtained from a colony maintained at the insectary of the Department of Entomology at North Carolina State University, Raleigh, NC. Larval instars were determined by measuring head capsule diameters with an ocular micrometer calibrated with a stage micrometer. Five to 10 individuals per developmental stage were analyzed for the presence of Manse-AT containing cells.

A. mellifera larvae were collected from colonies maintained at a research apiary at the University of Minnesota Duluth, Duluth, MN. Worker larvae of the fourth (L4) and fifth (L5) instars were distinguished by the differences in maximum diameter of their head capsules (Rembold et al., 1980). The fifth instar was divided into nine substages: small, medium, and large feeding stage larvae that were characterized by body weight (L5F1: 30–60 mg; L5F2: 61–110 mg; L5F3: 111–160 mg), early, intermediate and late spinning stage larvae that could be distinguished by checking the contents of their gut under a dissecting microscope (L5S1: entire gut filled with yellowish pollen mass; L5S2: only distal end of the gut filled with pollen mass; L5S3: gut completely void), and early, intermediate and late prepupae, which were classified by measuring the tibiotarsal length of the developing hind legs using an ocular micrometer (L5PP1: 1.40–1.99 mm; L5PP2: 2.0–2.6 mm; L5PP3: >2.6 mm) (Rachinsky and Hartfelder, 1990; Rachinsky and Feldlaufer, 2000). Mainly late fifth instar larvae were analyzed, since in workers, JH biosynthesis is low during the first half of the last instar and increases during the second half of this developmental stage (Rachinsky and Hartfelder, 1990). The following substages were included: L5F3, L5S1, L5S3, L5PP1, and L5PP3. Fifteen to 20 individuals per substage were analyzed for the presence of Manse-AT containing cells.