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# Development and steroid regulation of RFamide immunoreactivity in antennal-lobe neurons of the sphinx moth *Manduca sexta*

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**Summary**

**During metamorphosis, the insect nervous system undergoes considerable remodeling: new neurons are integrated while larval neurons are remodeled or eliminated. To understand further the mechanisms involved in transforming larval to adult tissue we have mapped the metamorphic changes in a particularly well established brain area, the antennal lobe of the sphinx moth *Manduca sexta*, using an antiserum recognizing RFamide-related neuropeptides. Five types of RFamide-immunoreactive (ir) neurons could be distinguished in the antennal lobe, based on morphology and developmental appearance. Four cell types (types II–V, each consisting of one or two cells) showed RFamide immunostaining in the larva that persisted into metamorphosis. By contrast, the most prominent group (type I), a mixed population of local and projection neurons consisting of about 60 neurons in the adult antennal lobe, acquired immunostaining in a two-step process during metamorphosis. In a first step, from 5 to 7 days after pupal ecdysis, the number of labeled neurons reached about 25. In a second step, starting about 4 days later, the number of RFamide-ir neurons increased within 6 days to about 60. This two-step process parallels the rise and fall of the developmental hormone 20-hydroxyecdysone (20E) in the hemolymph. Artificially shifting the 20E peak to an earlier developmental time point resulted in the precocious appearance of RFamide immunostaining and led to premature formation of glomeruli. Prolonging high 20E concentrations to stages when the hormone titer starts to decline had no effect on the second increase of immunostained cell numbers. These results support the idea that the rise in 20E, which occurs after pupal ecdysis, plays a role in the first phase of RFamide expression and in glomeruli formation in the developing antennal lobes. The role of 20E in the second phase of RFamide expression is less clear, but increased cell numbers showing RFamide-ir do not appear to be a consequence of the declining levels in 20E that occur during adult development.**

Key words: peptide, RFamide, hormonal regulation, development, olfactory system, *Manduca sexta*, immunoreactivity.

## Introduction

The antennal lobe (AL) is the first integration center for odor information in the insect brain and corresponds to the olfactory bulb of vertebrates (Strausfeld and Hildebrand, 1999; Eisthen, 2002). The building blocks of the AL and the olfactory bulb are spheroidal neuropil structures termed glomeruli, which in *M. sexta* and other holometabolous insects are formed during metamorphosis (Oland and Tolbert, 1996; Hildebrand et al., 1997; Salecker and Malun, 1999; Schröter and Malun, 2000). Each glomerulus consists of thousands of synapses between olfactory receptor neurons, local neurons and projection neurons. Local neurons are responsible for signal processing within and between glomeruli. The principle transmitter of local AL interneurons is γ-aminobutyric acid (GABA; Homberg and Müller, 1999), but immunostaining in a variety of species suggests that neuropeptides are also present in certain subpopulations of local neurons and might act as cotransmitters with GABA (Homberg and Müller, 1999).

Among the peptides found in AL neurons are members of the superfamily of FMRFamide-related peptides (FaRPs, Homberg et al., 1990; Homberg and Müller, 1999). Members of this family are small peptides of 4–18 amino acids ending with -RFamide at the C terminus (Greenberg and Price, 1992; Taghert, 1999; Orchard et al., 2001).

FMRFamide itself was first isolated as a cardioexcitatory peptide from the Venus mussel *Macrocalista nimbosa* (Price and Greenberg, 1977). Since then, FaRPs have been described in the nervous systems of all major animal phyla, including vertebrates (for reviews: general, see Greenberg and Price, 1992; cnidarians: Grimmelikhuijzen et al., 1996;

nematodes: Maule et al., 1996; Nelson et al., 1998; Li et al., 1999; molluscs: Santama and Benjamin, 2000; insects: Taghert, 1999; Orchard et al., 2001; Homberg, 2002; Nässel, 2002; vertebrates: Dockray et al., 1983; Wright and Demski, 1996; Perry et al., 1997). To date, a wide range of physiological effects of FaRPs has been demonstrated. In arthropods various neuromodulatory effects have been described on skeletal and visceral musculature (Worden et al., 1995; Skerrett et al., 1995; Orchard et al., 2001; Merte and Nichols, 2002). In the mollusc *Aplysia californica*, FMRFamide has inhibitory effects on sensory neurons and motoneurons mediating the gill and siphon withdrawal reflex and is involved in synaptic modification and learning (Small et al., 1992; Pieroni and Byrne, 1992; Peter et al., 1994; Wu and Schacher, 1994; Zhu et al., 1995; Sun et al., 1996; Santarelli et al., 1996; Belkin and Abrams, 1998; Keating and Lloyd, 1999). In vertebrates, FaRPs affect central serotonergic transmission (Muthal and Chopde, 1994, 1995; Muthal et al., 1997), opioid systems and pain regulation (Kavaliers, 1990; Vilim et al., 1999).

In the nervous system of *M. sexta* three different FaRPs have been identified (Manse FLRFamides: F10, F7G, F7D; Kingan et al., 1990, 1996). To date, various physiological activities on peripheral target organs such as skeletal muscles, gut and heart have been attributed to the three Manse FLRFamides (Kingan et al., 1990, 1996; Lee et al., 1998; Miao et al., 1998). All three peptides have an -SFLRFamide at the C terminus but different N-terminal extensions. Quantitative analysis of homogenates showed that the relative concentrations of the three peptides in various ganglia change during postembryonic development (Kingan et al., 1996; Miao et al., 1998). A transient decline of peptide levels in thoracic and abdominal ganglia and an accompanying increase in peripheral neurohemal sites (the transverse nerves) correlates with the time of ecdysis and suggests that *Manduca* FaRPs might be involved in modulation of skeletal and visceral muscles that facilitate ecdysis (Miao et al., 1998).

Recent cloning of the F10 gene revealed a single copy gene, and northern blot analysis showed a developmental regulation of F10 expression in the CNS (Lu et al., 2002). *In situ* hybridization revealed specific expression of the F10 gene in the brain and ventral nerve cord of *M. sexta*. In the brain, new neurons expressing the F10 gene during metamorphosis mainly belong to two cell clusters located in the optic (about 100 neurons) and in the antennal (about 12 neurons) lobes (Lu et al., 2002).

In this paper we describe the occurrence and hormonal regulation of RFamide immunoreactivity during development of the antennal lobe of *M. sexta*. Analysis of the cellular occurrence of FaRPs is the first step to understanding the role of this peptide family during metamorphosis of the AL. The presence of FaRPs at defined stages of AL development makes them candidate molecules for having developmental effects during these periods.

## Materials and methods

### *Animals*

*Manduca sexta* L. (Lepidoptera: Sphingidae) were raised on artificial diet under a long-day photoperiod (17 h:7 h L:D) at 26°C in walk-in environmental chambers (Bell and Joachim, 1978). Under these conditions the time required from hatching to pupal ecdysis is about 18 days, and the time from pupal to adult ecdysis about 20 days. The start of the wandering stage (W0) occurs 3–4 days into the fifth larval instar and is characterized by the appearance of a red pigment along the heart. The following days are referred to as W1–W4. At about noon of W2 the animals go into a quiescent prepupal stage. Pupal ecdysis occurs at W4 and the newly formed pupa is designated P0. Subsequent days of adult development are counted as P1–P20. Larvae and pupae were staged according to criteria described by Jindra et al. (1997) and Schwartz and Truman (1983). The criteria involve changes in structures that are either superficial or readily visible through the pupal cuticle under a dissecting microscope.

### *Immunocytochemistry*

Anti-RFamide antiserum raised in rabbit (#671, used at dilutions of 1:4 000 to 1:10 000) was kindly provided by Dr E. Marder (Brandeis University, USA). A polyclonal anti-GABA antiserum (#4TB, diluted 1:50 000) was kindly provided by Dr H. Dircksen (University of Bonn, Germany), and a monoclonal anti-synaptotagmin antibody raised in mouse (diluted 1:2500) was kindly provided by Dr K. Menon (Caltech, USA). The RFamide antiserum recognizes FMRFamide and FLRFamide peptides (Marder et al., 1987; Kingan et al., 1990), including the three FaRPs identified in *M. sexta* (Kingan et al., 1990, 1996; Miao et al., 1998). The specificity of the anti-GABA antiserum has been characterized by Homberg et al. (1999). Specificity of the anti-synaptotagmin antibody for *M. sexta* nervous tissue is described by Dubuque et al. (2001). The antibody against the ubiquitous synaptic vesicle protein synaptotagmin has been shown to label synapse-rich neuropil areas and can be used to label glomeruli through development (Dubuque et al., 2001).

Secondary antibodies, goat anti-rabbit or goat anti-mouse conjugated to Cy2, Cy3, Cy5 or horseradish peroxidase (all Jackson ImmunoResearch, Westgrove, PA, USA), were used at 1:300 dilution. After dissection in cold saline (Weevers, 1966), brains from various developmental stages of *M. sexta* were fixed in 4% formaldehyde in PBS (phosphate-buffered saline, pH 7.4) for 2 h at room temperature or overnight at 4°C. After fixation, brains were embedded in gelatin/albumin, postfixed overnight in 8% buffered formaldehyde, and cut at 40 μm with a vibrating blade microtome (Leica VT 1000S; Nussloch, Germany) in the frontal or horizontal plane. Sections were rinsed in 0.1 mol $l^{-1}$ Tris HCl (Sigma-Aldrich Chemie, Munich, Germany)/0.3 mol $l^{-1}$ NaCl (SST, pH 7.4) containing 0.1% Triton X-100 (SST-TX 0.1) for 1 h at room temperature, then preincubated for another hour with 5% normal goat or donkey serum (Jackson ImmunoResearch) in SST-TX 0.5

(SST containing 0.5% Triton X-100). Primary antibodies were diluted in SST-TX 0.5 with 1% normal goat or donkey serum. After incubation with the primary and secondary antisera, sections were rinsed 3 times 10 min in SST-TX 0.1 at room temperature. For double labeling, the anti-RFamide and the anti-synaptotagmin antibodies were applied simultaneously and, likewise, the corresponding secondary antisera. For double labeling using the anti-GABA and the anti-RFamide antiserum, which were both raised in rabbits, the anti-GABA antiserum was applied first as described above. Then Fab fragments raised in goat against rabbit immunoglobulins (1:50; Jackson ImmunoResearch, in SST-TX 0.5 including 1% normal donkey serum) were applied for 2 h at room temperature (protocol modified from Dianova, Hamburg, Germany). After washing 3 times for 10 min, secondary donkey anti-goat antiserum coupled to Cy3 was applied for 1 h at room temperature and, after washing (3 times for 10 min) the anti-RFamide antiserum and the corresponding secondary antiserum coupled to Cy2 were applied as described above. Horseradish peroxidase was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) using the glucose oxidase (Sigma-Aldrich) technique according to Watson and Burrows (1981). Sections were mounted on chromalaun/gelatin-coated microscope slides, dehydrated in ethanol, cleared in xylene and mounted in Entellan (Merck, Darmstadt, Germany).

### *Dextran application*

Crystals of biotinylated dextran (lysine-fixable, molecular mass 3000; Molecular Probes, Eugene, OR, USA) were placed on the cut ends of one antenna of immobilized prepupae (W2). The antennal stump was sealed with vaseline. The animal was kept in a humid chamber overnight at 4°C to allow the dextran to diffuse through the antennal nerve into its target area in the brain. The next day animals were dissected, and the brains were processed for immunocytochemistry as described above. Dextran was visualized using Cy2-coupled streptavidin (1:300, Jackson Immuno Research), which was applied for 1 h at room temperature.

### *Hormone manipulation*

20-hydroxyecdysone (20E, Sigma-Aldrich) was dissolved in saline (Ephrussi and Beadle, 1936) to a final concentration of 1 μg $\mu l^{-1}$. Pupae were chilled on ice for 2 min and then injected with 15 μg 20E $g^{-1}$ body mass (Schachtner et al., 1999). Control animals were injected with 15 μl saline $g^{-1}$ body mass. All injections were performed using 100 μl Hamilton syringes dorso-laterally into the pupal thorax. Wounds were sealed immediately with melted wax, and animals were returned to the walk-in environmental chambers. Animals were dissected 2–6 days later and processed according to the immunocytochemistry protocol described above.

### *Data processing*

Diaminobenzidine-treated sections were photographed using a Polaroid DMCe digital camera mounted on a Zeiss Axioskop (Jena, Germany). Images were imported into Adobe Photoshop 6.0 and annotated in Microsoft's PowerPoint or CorelDraw 10. Fluorescence was analyzed using a confocal laserscan microscope (Leica TCS sp2). Cell counts were performed at a magnification of ×400. We used two strategies to obtain numbers of labeled cell bodies in the lateral cell group. (1) For up to 35 cell bodies in total, we compared section by section to ensure that each cell was only counted once. (2) For more than 35 cells in total, we counted every stained cell body, including fragmented somata in each section, and used the Abercrombie correction factor to obtain real cell numbers (Abercrombie, 1946). For a section thickness of 40 μm and mean cell body diameter of 16.2 μm [obtained from measuring the diameters of 32 cell bodies in the lateral cell group in a total of four antennal lobes at stages P9, P10, P12 and pharate adult (P18)], the correction factor used was 0.712. Comparison of both counting methods in the left and right antennal lobes of a P10 and a P13 pupa resulted in a maximal difference of four cell bodies counted, which corresponded to a maximal difference of about 10%.

## Results

### *Identity of RFamide-ir neurons*

Based on morphological differences and different developmental time courses in the appearance of immunostaining, five types of RFamide-ir neurons could be distinguished in the AL of *M. sexta*.

Most immunostained neurons (type I) belonged to a mixed population of local interneurons and projection neurons in the lateral cell group (LC), as described by Homberg et al. (1990) (Figs 1A–C, 2). Type I cells gave rise to dense RFamide immunostaining in all glomeruli and in the coarse neuropil of the AL (Figs 1, 3). Fibers of type I cells also occurred in the outer antenno-cerebral tract, which carried their axons to higher brain centers (Fig. 1B; Homberg et al., 1990). Among the cell bodies in the LC we could not distinguish between local and projection neuron somata and thus decided to treat them collectively as type I.

A single neuron (type II) with a large cell body (diameter about 50 μm) in the LC had no arborizations within the AL but projected its neurite out of the AL (Fig. 1C). In some preparations we could trace the neurite into the tritocerebrum toward the subesophageal ganglion before it intermingled with numerous other RFamide-ir processes (Fig. 1D). Type III neurons consisted of two labeled cell bodies in the LC. Their neurites projected toward the AL neuropil but staining in the neurites faded out on their course to the AL neuropil so that projections in the AL could not be determined (Figs 1D; 3A). Up to stage P2, a single neurite (type IV) gave rise to a tree of arborization in the larval antennal center (LAC)/AL (Fig. 1D). This neurite entered the LAC/AL neuropil baso-laterally and in one preparation we could identify its cell body in a cell group below the LC (Fig. 1D). As we never observed a labeled neurite leaving the LAC/AL from the type IV arborizations, the neuron is most likely to be a larval local interneuron of the

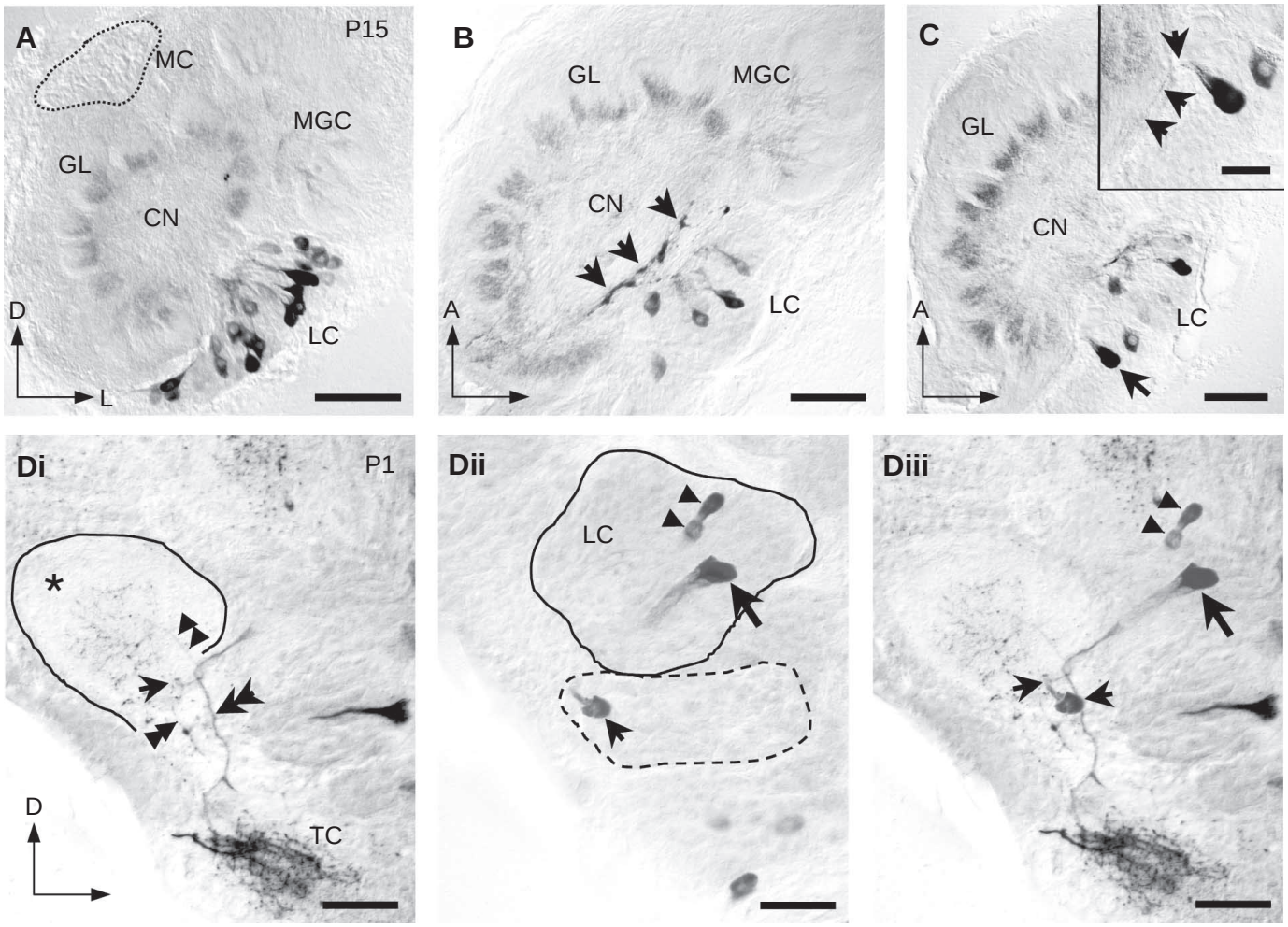


Fig. 1. Normarski images of 40 μm sections showing cell types I–V of the AL labeled with the RFamide antiserum. (A) Type I cells in the lateral cell group (LC) of a stage P15 AL. Staining within the glomeruli (GL) primarily results from type I neurons. No immunostaining is visible in the median cell group (MC). (B,C) Consecutive horizontal sections through a P13 AL. (B) In addition to glomerular staining, type I cell bodies give rise to stained fibers in the root of the outer antenno-cerebral tract (arrows). (C) Section ventral to B. The large cell body of the type II cell (arrow) has a posterior-medial position within the LC. Inset shows the primary neurite (arrows) leaving the AL without sending processes to the AL neuropil. (Di–iii) Type II–V neurons in a stage P1 pupa. Di and Dii are both superimposed images of two consecutive sections. Diii combines Di and Dii (total of four consecutive sections) with parts of the anterior laying cell groups omitted to better see the connections between cell bodies and neurites. The neurite (double arrow in Di) of the type II cell (large arrow in Dii, iii) leaves the AL towards the tritocerebrum (TC) while the neurite of the type V neuron (double arrowhead in Di) enters the AL. The neurite (arrowheads in Di) of the type IV neuron gives rise to processes that cover most of the developing AL area (solid outline in Di). The cell bodies of type II, III (arrowheads in Dii, iii), and IV neurons (small arrows in Dii, iii) are located anteriorly to the AL neuropil. Small arrow in Di, Dii points toward the connection site of the type IV neuron with its cell body. The type IV cell body lies in a cell group (broken outline in Dii), which is different from that of type II and III neurons (solid outline in Dii). A, anterior; D, dorsal; L, lateral; CN, coarse neuropil; MGC, male specific macroglomerular complex; TC, tritocerebrum. Scale bars, 100 μm (A–C); 50 μm (inset in B, Di–iii).

AL. Another single neuron (type V) gave rise to a sparse meshwork of varicosities, mainly in basal parts of the AL. The location of its soma was not evident, but was probably outside the AL, since a neurite connecting to the meshwork from outside the AL could clearly be observed in many preparations (Figs 1D, 3B,E,F).

### *Time course of RFamide immunoreactivity in the developing AL*

Cell bodies of type I neurons were not labeled with the RFamide antiserum in pupal stages earlier than P5 (*N*=11). At stage P5, four (from two animals) of 11 ALs analyzed still showed only type II and III cells. The numbers of type I neurons increased from P5 to P7/8 in a first step to about 25 strongly labeled cell bodies (Fig. 4). This number remained relatively constant up to P10/11 and increased from P11 to P16 in a second step to about 60 cells (Figs 3, 4). As early as P5 we observed faint RFamide immunostaining in the developing AL neuropil. With the beginning of synaptogenesis and the appearance of protoglomeruli and

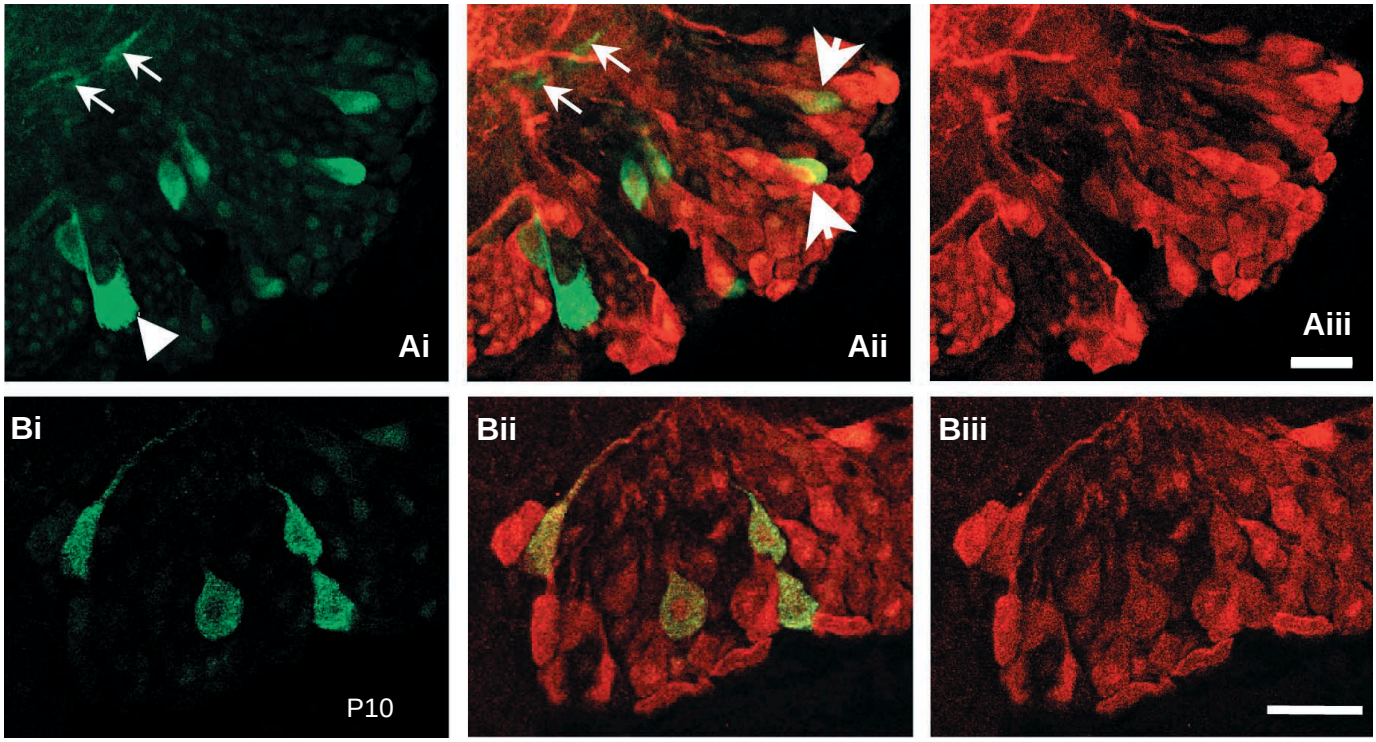


Fig. 2. Confocal images of 40 μm sections showing double immunostaining in the lateral cell group (LC) of P10 antennal lobes with antisera against RFamide (green, Cy2; Ai, Bi) and GABA (red, Cy3; Aiii, Biii). (Aii, Bii) Overlays of the respective immunostainings in Ai/Aiii and Bi/Biii. (A) RFamide-ir cell bodies in the LC, of which two show double labeling with the GABA antiserum (large arrows in Aii). The arrowhead (Ai) points to the large type II cell body, which like the root of the outer antenno-cerebral tract (small arrows) shows only RFamide and no GABA immunostaining. (B) In another section all type I cells colocalize RFamide and GABA immunoreactivity. Scale bars, 40 μm.

glomeruli at stage P7/8 (Dubuque et al., 2001) the relatively weak staining became confined to the basal parts of all developing glomeruli (Fig. 3C). In later stages (from P12) immunostaining in the glomeruli strongly increased in intensity and extended finger-like protrusions from basal to more distal parts of each glomerulus (Figs 3D,E). RFamide labeling in the outer antenno-cerebral tract (OACT) occurred around pupal stage P7/8. Double immunostaining with the anti-RFamide- and an anti-GABA antiserum at stage P10 revealed about ten (10±1, *N*=3) cell bodies in LC, which were only immunopositive for RFamide and not for GABA, suggesting that these ten cells are the source of the fibers in the OACT (Fig. 2).

Type II and III neurons could already be identified in late fifth feeding instar larvae (the earliest time point examined, *N*=4 animals) and could be followed through wandering stages (*N*=7 animals) into early pupal stages (Figs 3A, 4). The type II neuron could be identified in all developmental stages and in the adult owing to its large size and its position in the LC. The two type III neurons intermingled from P5/6 with the increasing numbers of type I neurons and could not be distinguished from them during further development.

The arborization pattern of the type IV neuron dramatically changed during metamorphosis. From the larva up to pupal stage P2/3 the arborizations in the LAC and in the developing AL were strongly labeled (Fig. 1D). In the following two stages the arborizations were reduced and had completely disappeared from the AL by stage P4 (Fig. 3B).

The varicose meshwork of the type V neuron in basal regions of the AL was found in fifth instar larvae up to the adult (Figs 1D, 3). During and after glomeruli formation (starting at P7/8), arborizations of type V neurons extended into distal areas of the glomeruli, which are densely packed with endings of incoming olfactory receptor neurons, while the projections of type I neurons were confined to the basal part of the glomeruli (Fig. 3C). Up to P12/13, staining of the type V meshwork was much more intense than that of the glomerular arborizations originating from type I cells (Fig. 3C). In later stages and in the adult, however, the staining intensity of type I neurons reached the intensity level of the type V meshwork, which was therefore hardly distinguishable from the type I arborizations (Fig. 3D).

### *Hormone manipulation*

The increasing number of RFamide-ir type I cell bodies in the LC parallels the increasing titer of 20-hydroxyecdysone (20E) in the hemolymph from P4 to P9 (Fig. 4; Warren and Gilbert, 1986). To test whether 20E is responsible for the increasing number of type I neurons, we injected 15 μg $g^{-1}$ body mass of 20E into the hemolymph of stage P1 pupae (Schachtner et al., 1999). Pupae were dissected 2–8/9 days later and processed for immunocytochemistry. Cell

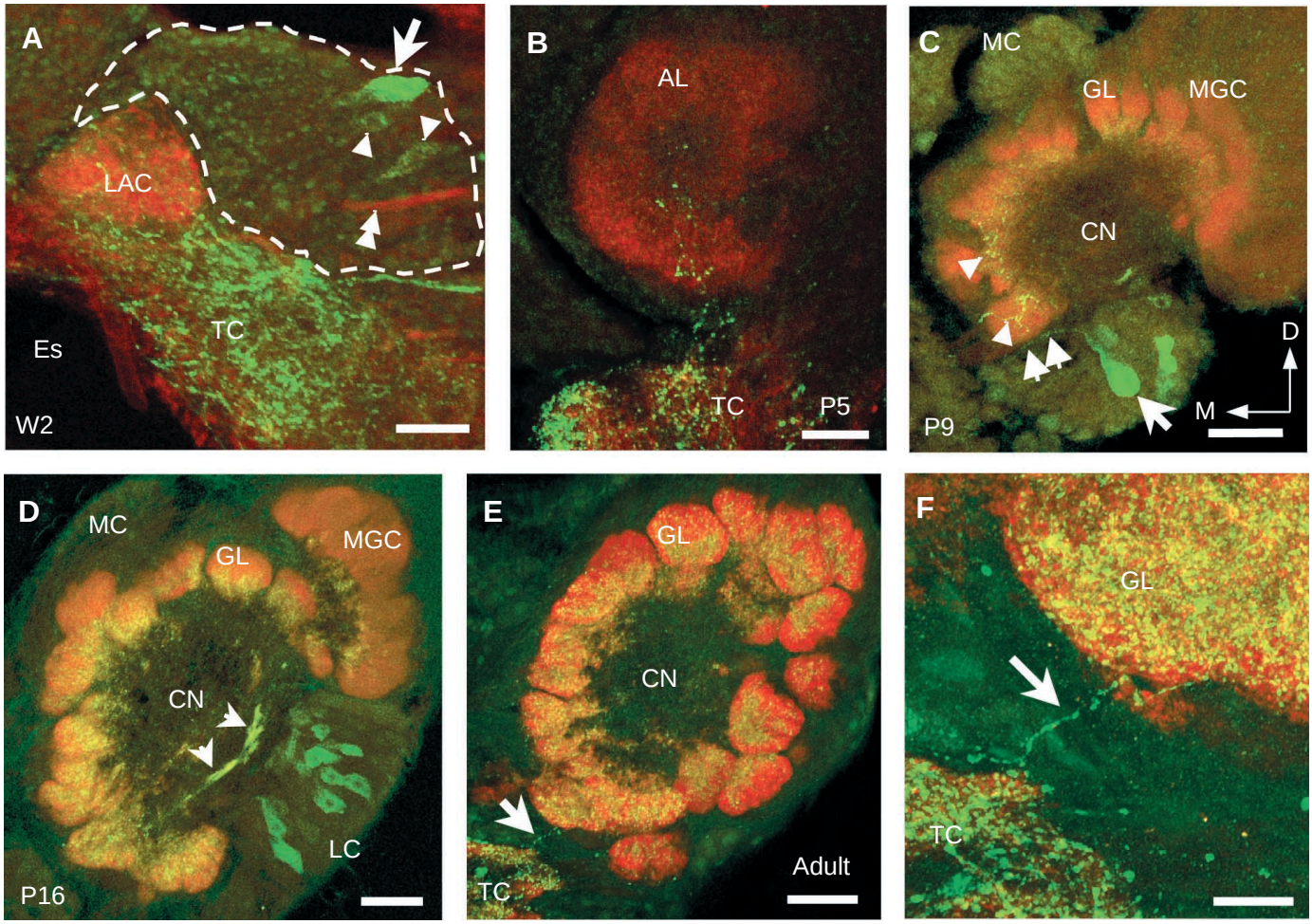


Fig. 3. Confocal images of 40 µm sections showing RFamide immunoreactivity (green, Cy2) during AL development. (A) Area of the larval antennal center (LAC) in a wandering larva (W2, 2-day-old wandering stage shortly before entering the prepupal stage). Within the developing lateral cell group (LC, encircled area) type II (arrow) and III (arrowheads) somata are stained. The LAC is labeled (red) by dextran backfill of the antennal nerve. The double arrowhead marks a dextran-labeled axon bundle coming from the larval antenna. (B–F) Double immunolabeling with the RFamide antiserum (green, Cy2) and the synaptotagmin antibody (red, Cy5). (B) Anterior section through the developing AL showing the type V neuron entering the AL from the tritocerebrum (TC). (C) In stage P9 developing glomeruli (GL) can be distinguished by synaptotagmin immunostaining. RFamide immunostaining in basal parts of the developing glomeruli is weak compared to staining of the type V arborizations (arrowheads) and to staining intensity in stages later than P12/13 (see D–E). In the LC the large type II cell (large arrow) with its neurite leaving the AL (smaller arrows) and smaller type I cells are labeled. (D) Frontal section through posterior parts of a stage P16 AL with strongly labeled type I cells, strong labeling in the glomeruli (GL) and intensely labeled fibers in the root of the outer antenno-cerebral tract (arrows). (E) Oblique section showing typical labeling of glomeruli in an adult AL. The type V cell enters the AL neuropil from the TC (arrow). (F) Magnification from E showing the neurite of the type V cell entering the AL neuropil. A–E, frontal sections; orientation bars in C (D, dorsal; M, medial) also apply to A–D; CN, coarse neuropil; Es, Esophagus; MC, median cell group; MGC, macroglomerular complex. Scale bars, 40 µm (A,B); 80 µm (C–E); 20 µm (F).

counts in the LCs of these animals already showed slightly higher numbers of RFamide-ir cell bodies 2 days after 20E injection (P3, Fig. 5A). 3 and 4 days after injection (P4, 5) the numbers of RFamide-ir cells had increased to a level that during normal development was reached 2 days later (Fig. 5A). 5–8/9 days after injection (P6–P9/10) the increase of LC cell numbers in 20E treated *versus* control animals was even more marked, corresponding to a difference of at least 5 days (Fig. 5A, compare with Fig. 4). In the 20E injected animals no obvious plateau niveau in LC cell numbers occurred (Fig. 5A). Instead, the numbers of RFamide-ir cells steadily increased.

Double labeling of vibratome sections of 20E injected animals with the RFamide antiserum and an antibody against synaptotagmin (Dubuque et al., 2001) was used to determine the developmental stage of glomeruli formation. By 3 days after 20E injection we found an advancement of glomerulus formation to a stage P7/8 AL, which is determined by the beginning of glomeruli formation (Fig. 6B, Dubuque et al., 2001). Compared to the 2 days advancement in the

development of RFamide immunostaining seen 3 days after hormone injection, formation of glomeruli was accelerated even more and was 3–4 days ahead of normal development.

Interestingly, 3 days after hormone treatment, the basal type V meshwork seemed to contain much more branching than was normally observed around this time of development (Fig. 6C). The large type II neuron did not change its arborization pattern after 20E treatment.

The second increase in the number of type I cells between P11 and P16 (Fig. 4) coincided with the decreasing 20E hemolymph titer (Warren and Gilbert, 1986). To test whether the falling 20E titer might cause the increase in RFamide-ir LC neurons, we injected 20E (15 μg $g^{-1}$ body mass) into P9 and P10/11 pupae. After 6/7 days (P15/16, P9 injection), 3/4 (P14, P10/11 injection) and 5/6 days (P16, P10/11 injection) the animals were dissected and subjected to immunocytochemistry. As judged by external developmental markers such as scale pigmentation on the thorax or on the wings (Schwartz and Truman, 1983; Jindra et al., 1997), the hormone treatment delayed metamorphic development for 23 days, respectively (inset in Fig. 5B). However, counts of RFamide-ir cells in LC did not reflect this external developmental delay (Fig. 5B). Also, the developmental state of the glomeruli revealed no obvious differences between the ALs of hormone-treated animals and control animals, as determined by synaptotagmin labeling (Dubuque et al., 2001).

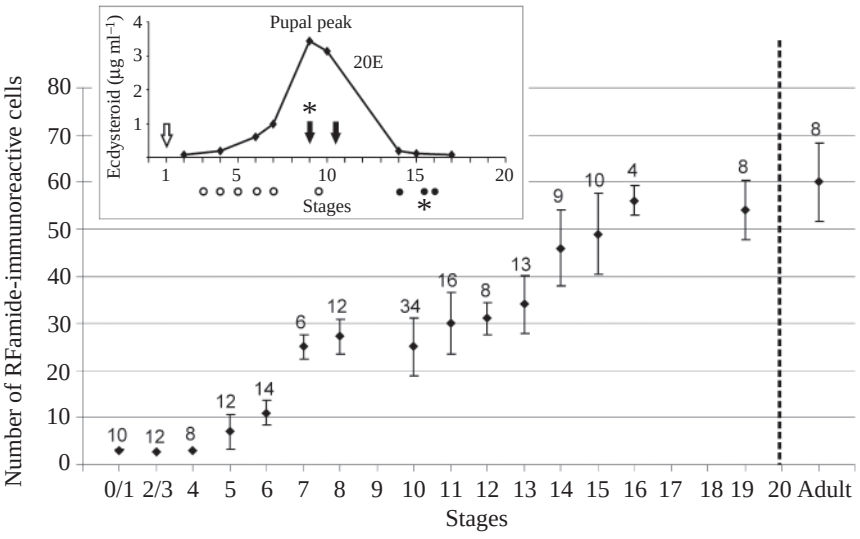


Fig. 4. Developmental time course of numbers of RFamide-ir cell bodies in the lateral cell group. Acquisition of RFamide immunostaining in AL neurons occurs in two phases. A first increase from stage P5 to P7/8 results in about 25 cells. The second increase in cell number (up to about 60 cells) occurs from P11/12 to P16. Each data point indicates mean ± S.D. The number of antennal lobes is indicated at each stage; stages P0+P1 and P2+P3 were pooled. The three cells at stages P0 to P4 are the type II and III neurons. The dotted line indicates adult eclosion at stage P20. Inset shows hemolymph concentrations of 20E during the pupal–adult transition as reported by Warren and Gilbert (1986). The open and filled large arrows mark the times of 20E injection at P1, P9 (asterisk) and P10/11, respectively, the open and filled circles (asterisk after P9 injection) mark the times when the injected animals were killed and processed for immunocytochemistry (see Fig. 5).

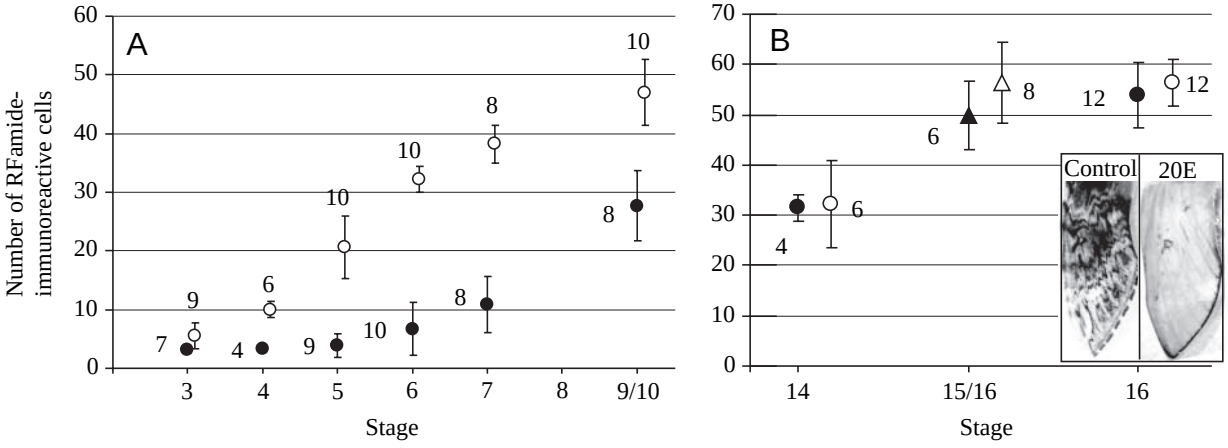


Fig. 5. Numbers of RFamide-ir neurons in the AL of *M. sexta* after hemolymph injection of 20E at P1 (A), P9 and P10/11 (B) and dissection several days later (see inset in Fig. 4). (A) Animals were injected with 20E at stage P1 and dissected 2 (P3) to 8/9 days (P9/10) later. Hormone-treated animals (open circles) showed a progressive increase in the numbers of RFamide-ir neurons compared to controls (filled circles) injected with saline. (B) Injections of 20E at P9 or P10/11 and dissection 6/7 days later (P9, open triangles) or 3/4–5/6 days later (P10/11, open circles) resulted in cell numbers that were not significantly different from the control animals (filled circles or triangles). Values are means ± S.D. The number of Als is shown for each stage. Inset in B shows wing pigmentation 6 days after injection of saline (control) or 20E at stage P10/11.

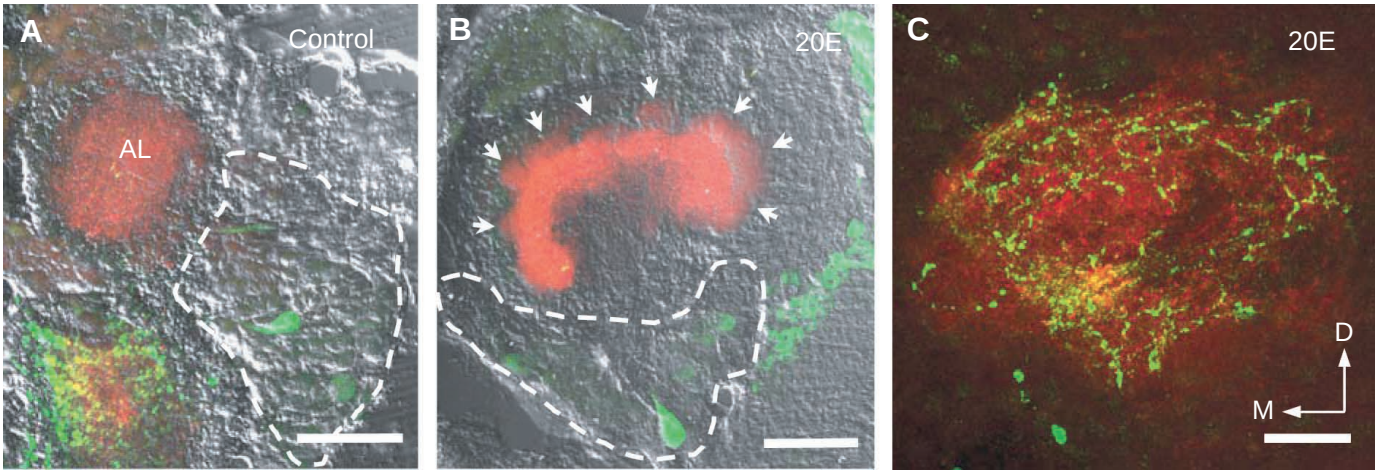


Fig. 6. Confocal images of 40 µm sections showing RFamide immunoreactivity (green, Cy2) and synaptotagmin immunoreactivity (red, Cy5) in antennal lobes (ALs) after hemolymph injection of 20E or after injection of the vehicle alone at stage P1 and dissection 3 days later at pupal stage P4. In A and B, additional Normarski images are shown (gray). (A) After injection of the vehicle alone ALs were indistinguishable from ALs of untreated animals at stage P4. In the lateral cell group (broken outline) two cell bodies are labeled. (B) 3 days after 20E injection the number of RFamide-ir cells in the LC (broken outline) increased to levels normally observed about 2 days later (see Fig. 5A). The glomerular pattern reached a stage normally observed at pupal stage P7/8 (Dubuque et al., 2001). Arrows point to developing glomeruli. (C) Posterior section through an AL showing a dense meshwork of strongly RFamide-ir arborizations, which is normally not observed in P4 or any other pupal stage. Frontal sections; scale bars, 80 µm (A,B); 40 µm(C). M, medial; D, dorsal.

## Discussion

### *Specificity of the RFamide antiserum*

The polyclonal RFamide antiserum used in this study has been shown to recognize peptides ending with -RFamide (Marder et al., 1987; Kingan et al., 1990). In *M. sexta*, three C-terminally extended FLRFamides, termed Manse-FLRFamides, have been identified (Kingan et al., 1990, 1996) and all are recognized by the antiserum used (Miao et al., 1998). Low levels of cross-reactivity with allatotropin and molluscan small cardioactive peptide B have been observed in ELISAs, but should not be seen in the less sensitive method of immunocytochemistry (Miao et al., 1998). A recent study by Lu et al. (2002) revealed that one of the three Manse-FLRFamides in *M. sexta* (F10) is newly expressed in at least 12 AL neurons during metamorphosis. Lu et al. (2002) did not exactly describe the location of these 12 neurons, but since RFamide-ir somata were almost exclusively found in the LC, these cells are most likely to be members of the LC. From pupal stage P16 on we found a total of about 60 RFamide-ir cells in LC. This suggests that more than one Manse-FLRFamide is present in LC neurons and that immunoreactivity in almost 50 LC neurons may originate from expression of genes other than the F10 gene. Candidates accounting for the additional RFamide immunoreactivity in LC are the Manse FLRFamides F7G and F7D and perhaps other FaRPs deriving from yet-unidentified genes.

### *RFamide immunoreactivity in larval and in adult-specific neurons*

The antennal lobe (AL) of *M. sexta* is newly built during metamorphosis. Its cellular components (local interneurons and projection neurons) derive from a few neuroblasts, which produce neurons during all larval instars up to pupal stage P3 when the last AL neurons are born (Hildebrand et al., 1997). In addition, an unknown number of interneurons of the larval antennal center (LAC), the progenitor of the AL in the larva, survives through metamorphosis and becomes part of the adult AL (Homberg and Hildebrand, 1994).

We describe RFamide immunoreactivity in five different types of AL neurons. Types II–V are already present in the fifth instar larva (L5). Of these neurons types II and V could be unequivocally followed into adulthood. Interestingly, neither in the larva nor in the adult did the large type II neuron project into the LAC/AL neuropil. Thus the type II neuron might not be directly involved in olfactory signal processing in the larva or in the adult. The neuron sends its primary neurite toward the subesophageal ganglion and, therefore, is not identical with the single serotonin-immunoreactive neuron of the AL, which extends its primary neurite to the protocerebrum (Kent et al., 1987). The type V neuron very likely has its cell body outside the AL and might thus account for a peptidergic centrifugal neuron. Due to the morphological changes observed during AL development, the type V meshwork clearly seems to be remodeled to fit the requirements of the adult AL. Also, the pattern of the type V meshwork seems to be under hormonal control as early 20E injection leads to a much denser meshwork than is normally observed in any of the stages examined (Fig. 6C). Immunostaining in type III neurites typically fades out after a short distance, possibly owing to low amounts of antigen, so that we could never observe whether they were connected to the LAC/AL neuropil. The characteristic arborization of the type IV neuron gradually

disappears from the developing AL neuropil after stage P2/3, which could mean that the arborizations are retracted during early metamorphosis. A later regrowth could not be detected, but would probably be masked by the increasing arborizations from type I neurons.

Type I neurons are the most prominent RFamide-ir cell group, consisting of about 60 neurons in the adult LC. Homberg et al. (1990) counted about 80 Rfamide-positive neurons in paraffin and in vibratome sections of adult ALs. The difference of about 20 cells may be caused by different counting methods (counts of nuclei by Homberg et al. *vs.* counts of somata in this study) or by differences in the specificities of the two antisera used in the two studies.

From double labeling experiments with antisera against RFamide and GABA, Homberg et al. (1990) concluded that about 20 RFamide-ir cells in LC might be projection neurons responsible for the staining in the outer antenno-cerebral tract (OACT) and in the isthmus of the AL. RFamide immunoreactivity in the root of the OACT occurred as early as stage P7/8. Double labeling with the RFamide- and the GABA antiserum in stage P10 ALs revealed RFamide- but not GABA immunostaining in about ten somata in LC (including the type II neuron) and in the OACT. These RFamide-ir cells (of a total of about 25 RFamide cells) that did not show additional GABA immunoreactivity were probably the projection neurons, which gave rise to RFamide immunostaining in the OACT. This suggests that throughout metamorphosis, acquisition of RFamides in local and projection neurons of the AL occurs in parallel and not sequentially.

Judged from the acquisition of RFamide immunostaining during metamorphosis, type I neurons are adult-specific neurons that are newly born during postembryonic development to serve functions in the adult (Truman, 1996a). In a few examples, however, it has been demonstrated that neurons can change their peptide identity (Loi and Tublitz, 1993; Tubliz and Loi, 1993; Witten and Truman, 1996). This raises the possibility, that some of the type I neurons could be larval neurons that change their peptide identity during metamorphosis.

### *Developmental regulation of RFamide immunoreactivity*

The developmental increase in the number of type I RFamide-ir cells showed three phases; a rising phase from P5 to P8, a plateau phase from P8 to P12, and a second rising phase from P12 to P16 (Fig. 4). This suggests that two populations of neurons acquire RFamide immunostaining, possibly by two developmental mechanisms, at different times of metamorphosis. The time course of RFamide-ir cell numbers shows two parallels with that of the adult 20E peak in the hemolymph (Fig. 4; Warren and Gilbert, 1986). The first increase in numbers of RFamide-ir neurons parallels the increasing 20E titer, whereas the second increase in cell numbers coincides with a decrease of the 20E titer.

The steroid hormone 20E acts on target cells by binding to a heterodimeric nuclear receptor consisting of the ecdysone receptor (EcR) itself and ultraspiracle, an orphan receptor (Riddiford et al., 2000). In *M. sexta* and *D. melanogaster* three EcR isoforms are known, and seem to correlate with different phases of neuronal development (Truman et al., 1994; Truman, 1996b). Many studies on 20E action have led to the assumption that steroidal fluctuations in combination with different EcR receptor isoforms can orchestrate developmental events during metamorphosis (Thummel, 1996; Truman, 1996a).

Experimentally shifting the onset of the pupal 20E peak by 20E injection to an earlier developmental time point resulted in the precocious appearance of RFamide-ir cells (Fig. 4A). This result clearly demonstrates a regulatory role of the 20E rise for the first phase of RFamide expression in the AL. Interestingly, the increase in cell numbers in 20E-injected animals did not show a plateau phase as observed in untreated animals (compare Figs 4 and 5A). This could suggest an inhibition of RFamide expression occurring during normal development between P8 and P12, which is absent after early 20E injection. On the other hand the lack of a plateau phase could simply reflect the fact that a single hormone injection does not mimic the pupal ecdysteroid peak as well as a continuous hormone infusion might do (Lehman et al., 2000). Furthermore, synaptotagmin staining revealed that not only the presence of RF-amides, but also the formation of glomeruli, occurred earlier than during normal development. Thus our results strongly suggest an important role of 20E in organizing the development of the AL with respect to glomeruli formation and transmitter acquisition.

The rising phase of the pupal 20E peak is associated with promotion of development, whereas preventing the declining phase seems to retard development (Schwartz and Truman, 1983; Truman, 1996a). Accordingly, experiments that prolonged the 20E peak into later developmental stages delayed pupal development, as judged by external developmental markers such as scale pigmentation on thorax and wings (inset Fig. 5B). However, the numbers of RFamide-ir neurons in the LC did not differ between treated and control animals (Fig. 5B). Thus, enhanced 20E levels at later developmental times did not prevent the second increase in RFamide-ir cell numbers. This result suggests that the decreasing 20E titer has no direct consequences on the numbers of RFamide-ir cells and implies the involvement of other unknown mechanisms in the second phase of FaRP acquisition.

Effects of 20E on metamorphic development of the nervous system of *M. sexta* have been shown in several studies. The pupal 20E peak regulates the fusion of thoracic and abdominal ganglia (Amos et al., 1996), it controls cell proliferation during genesis of the optic lobes and the retina but also programmed cell death of optic lobe neuroblasts (Champlin and Truman, 1998a,b, 2000). Specifically, 20E also regulates the pupal expression of tyramine β-hydroxylase, an essential enzyme for octopamine biosynthesis (Lehman et al., 2000). We recently showed that 20E injections early in metamorphosis lead to elevated concentrations of the second messenger molecule cyclic guanosine monophosophate in LC neurons, which

during normal development does not occur before stages P7/8 (Schachtner et al., 1998, 1999). Fluctuations of 20E other than the pupal peak have also been shown to be involved in aspects of nervous system development including reorganization of neuronal networks (Levine et al., 1986; Levine, 1989; Truman and Reiss, 1995) and regulating alterations in neuroactive substances (Loi and Tublitz, 1993; Tublitz and Loi, 1993; Witten and Truman, 1996; Žitňan et al., 1999). Žitňan et al. (1999) even established in an elegant study that rising 20E levels can directly influence expression of a gene that encodes for two peptides which are needed for preecdysis and ecdysis behavior in *M. sexta*.

*Possible roles of FaRPs during nervous system development*

Several recent studies suggest possible roles of neuropeptides during neuronal development. During embryonic and larval development of the lobster stomatogastric nervous system, defined neurons express various neuropeptides (e.g. FLRFamides; Fenelon et al., 1998, 1999; Kilman et al., 1999). These neuropeptides are thought to play a role in activity dependent tuning of intrinsic and synaptic properties of the developing stomatogastric ganglion pattern generator (Marder and Richards, 1999). In molluscs, neurons labeled with an FMRFamide antiserum occur very early in embryonic development and it is speculated that they might be involved in neurogenesis (Voronezhskaya and Elekes, 1996; Croll, 2000). Recent data from the developing or regenerating mouse olfactory epithelium demonstrated a role of two neuropeptides (neuropeptide Y and pituitary adenylate cyclase-activating polypeptide) in initiating proliferation of basal cells (Hansel et al., 2001a,b). In vertebrates there is increasing evidence that peptides exert trophic actions during embryonic development (Strand et al., 1991; De Felipe et al., 1995; Hökfelt et al., 2000). FaRPs also play a role in learning and neuronal plasticity, which are thought to imply similar mechanisms as used during development (Carew et al., 1998). In *Aplysia* FMRFamide induced long-term depression (LTD) leads to downregulation of a neuronal cell adhesion molecule (apCAM; Peter et al., 1994). During AL development in *M. sexta*, fasciclin II, an insect homologue of apCAM is expressed by developing olfactory receptor neurons and serves a role in guiding the growing axons to the correct glomeruli (Rössler et al., 1999; Higgins et al., 2002).

The present study revealed for the first time in an insect an exact analysis of the cellular appearance of neuropeptide expression during the development of a defined brain area, the AL of *M. sexta*. We showed by using immunocytochemistry that FaRPs occur in several types of AL neurons at certain times of metamorphosis. Manipulating the pupal 20E titer revealed that one group of AL neurons expresses FaRPs under the developmental control of 20E. Furthermore, the timing of glomerular formation is under the control of the same 20E peak. The parallel time courses of certain phases of AL development with the acquisition of RFamide immunoreactivity are consistent with the hypothesis that RFamides might be involved in certain aspects of AL development.

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